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VOLUME 53(1) JANUARY 1975

British Journal of Pharmacology

page

Systematic Pharmacology

3 MURNAGHAN, M.F. The effect of sympathomimetic amines on the ventricular fibrillation threshold in the rabbit isolated heart. (SP1, AP1, DM1)

11 GRAYSON, J. & SCOTT, C. The action of nitroglycerine and dipyridamole in normal and ischaemic dog heart. (SP1)

21 METCALF, G. & THOMPSON, J.W. The effect of various amine-depleting drugs on the fever response exhibited by rabbits to bacterial or leucocyte pyrogen. (SP2, AP1)

29 BEVAN, P., BRADSHAW, C.M. & SZABADI, E. The effect of tricyclic antidepressants on cholinergic responses of single cortical neurones. (SP2, DM2)

37 MARLEY, E. & WHELAN, JENNIFER E. Some central effects of 5-hydroxytryptamine in young chickens at and below thermoneutrality. (SP2, DM2)

Autopharmacology

43 ADLER-GRASCHINSKY, EDDA & LANGER, S.Z. Possible role of a β-adrenoceptor in the regulation of noradrenaline release by nerve stimulation through a positive feed-back mechanism. (AP1, PK3, DM2)

51 KRAFT, E. & ZIMMERMAN, B.G. Influence of histamine H_1- and H_2-receptor blockers on sympathetic vasodilator and vasoconstrictor responses in canine paw. (AP2, SP1)

59 CROCKER, ANN D. & WILSON, K.A. A further investigation into the energy dependence of angiotensin II-induced contractions of isolated smooth muscle preparations. (AP3, DM1)

67 JOHNS, E.J., RICHARDS, H.K. & SINGER, BERTHA. Effects of adrenaline, noradrenaline, isoprenaline and salbutamol on the production and release of renin by isolated renal cortical cells of the cat. (AP3, DM2)

75 LEME, J. GARCIA & SCHAPOVAL, ELFRIDES E.S. Stimulation of the hypothalamo–pituitary–adrenal axis by compounds formed in inflamed tissue. (AP3)

Miscellaneous

85 ATKINSON, D.C. & HICKS, R. The possible occurrence of endogenous anti-inflammatory substances in the blood of injured rats. (M)

Pharmacokinetics

93 LOVELESS, A.H. & MAXWELL, D.R. The prevention by sulphydryl compounds of the toxicity in the cat of 2,6-dimethoxyphenol and its morpholinopropionyl ester. (PK2)

99 LULLMANN, H., PETERS, T. & RAVENS URSULA. Studies on the kinetics of $[^3H]$-ouabain uptake and exchange in the isolated papillary muscle of the guinea-pig. (PK3, PK1)

109 FOSTER, R.W. & O'DONNELL STELLA R. Evidence that adrenergic nerves are responsible for the active uptake of noradrenaline in the guinea-pig isolated trachea. (PK3, AP1)

Drug Mechanisms

113 NORTHOVER, B.J. Effect of anti-inflammatory drugs on the membrane potential of vascular endothelial cells *in vitro*. (DM1)
121 BOULLIN, D.J., GRAHAME-SMITH, D.G., GRIMES, R.P.J. & WOODS, H.F. Inhibition of 5-hydroxytryptamine-induced human blood platelet aggregation by chlorpromazine and its metabolites. (DM1)
127 HÅKANSON, R., HEDENBRO, J., LIEDBERG, G., REHFELD, J.F. & STADIL, F. Activation of histidine decarboxylase by H_2-receptor blockade: mechanism of action. (DM2, AP2, AP3)
131 KOSTERLITZ, H.W. & WATERFIELD, ANGELA A. An analysis of the phenomenon of acute tolerance to morphine in the guinea-pig isolated ileum. (DM2, AP1)

Pharmacometrics

139 BARLOW, R.B. Use of the logistic function for the calculation of dose-ratios and potency ratios. (PM)
143 PEARSON, J.D.M. & SHARMAN, D.F. The estimation of 3,4-dihydroxyphenylacetic acid, homovanillic acid and homo-isovanillic acid in nervous tissue by gas–liquid chromatography and electron capture detection. (PM, SP2)

SHORT COMMUNICATIONS

149 ELLIOTT, ANNIE B. The effect of oxytocin on blood vessels of cremaster muscle of the rat. (AP3)
152 GULATI, O.D. & SIVARAMAKRISHNA, N. Kinetics and some characteristics of uptake of noradrenaline by the human umbilical artery. (PK3, AP1)

Systematic Pharmacology
SP1 Cardiovascular system
SP2 Central nervous system
SP3 Other physiological systems

Pharmacokinetics
PK1 Storage, distribution, fate
PK2 Synthesis, metabolism
PK3 Uptake, release

Autopharmacology
AP1 Adrenergic, cholinergic transmission
AP2 Histamine, kinins, prostaglandins, etc.
AP3 Hormones

Drug Mechanisms
DM1 Molecular, cellular, biochemical, biophysical
DM2 Receptors, antagonism, potentiation, tolerance

CP **Clinical Pharmacology, Experimental Therapy**
CT **Chemotherapy**
M **Miscellaneous**

PM **Pharmacometrics**
PP **Psychopharmacology**
TX **Toxicology**

Br. J. Pharmac. (1975), 53, 3-9

THE EFFECT OF SYMPATHOMIMETIC AMINES ON THE VENTRICULAR FIBRILLATION THRESHOLD IN THE RABBIT ISOLATED HEART

M.F. MURNAGHAN

Department of Physiology, University College, Dublin, Ireland

1 The ventricular fibrillation threshold (VFT) was measured in the isolated heart of the rabbit perfused via the aorta with McEwen's solution at 37° C by applying a single 10 ms pulse of current during the vulnerable period of late systole. The arrhythmia induced was either fibrillation or a rapid tachycardia.

2 The catecholamines adrenaline, noradrenaline and isoprenaline, but not dopamine, when infused at rates which produced similar moderate effects on cardiac rate and force, significantly lowered the VFT; it was reduced slightly more by adrenaline than by the other two. Phenylephrine and methoxamine were ineffective. Only those sympathomimetic amines which lowered the VFT also shortened the vulnerable time, i.e. minimal time after the R-wave of the ECG at which the pulse had to be applied to induce the arrhythmia.

3 The lowering effect of adrenaline on the VFT was not influenced by phentolamine but was blocked by propranolol and pindolol.

4 Chloroform potentiated the lowering effect of adrenaline, but not that of isoprenaline, on the VFT. Carbachol did not alter the effect of adrenaline on the VFT.

5 The results indicate that adrenaline, noradrenaline and isoprenaline lower the VFT by a direct action on the cardiac musculature and that this effect is mediated via β-adrenoceptors.

Introduction

It has been well authenticated that catecholamines may induce cardiac arrhythmias and occasionally ventricular fibrillation. Some workers have suggested that an increased vagal discharge to the heart may play a significant role in the induction of ventricular arrhythmias and fibrillation by adrenaline (Riker, Depierre, Roberts, Roy & Reilly, 1955). Depression of sino-atrial node activity (Roberts & Baer, 1960) and of atrio-ventricular nodal conduction (Dresel, 1962) are believed to permit the emergence of ventricular pacemakers due to the loss of driving dominance by the higher pacemaker. On the other hand acetylcholine (MacLeod & Reynolds, 1964) and methacholine (Hoff & Nahum, 1934) have been shown to suppress or reduce ventricular adrenaline-induced arrhythmias and vagal stimulation to increase the ventricular fibrillation threshold (Kent, Smith, Redwood & Epstein, 1973). Various anaesthetics, e.g. chloroform (Oliver & Schäfer, 1895; Levy & Lewis, 1911-12), cyclopropane (Meek, Hathaway & Orth, 1937), halothane (Andersen & Johansen, 1963) and other agents, e.g. benzol (Wégria & Nickerson, 1943) sensitize the dog and cat heart muscle so that an intravenous injection of adrenaline or noradrenaline induces ventricular fibrillation in a high proportion of trials. However, isoprenaline, invariably failed to induce ventricular fibrillation under similar circumstances (Garb & Chenoweth, 1948) but did induce a ventricular tachycardia (Katz, 1965).

In the studies quoted where the anaesthetic agent sensitized the heart to the sympathomimetic amine, the experiments were carried out on the anaesthetized animal where merely the incidence of a ventricular tachycardia and/or fibrillation was recorded after a chosen dose of the sympathomimetic amine which gave an all-or-none response. In all these studies the whole animal was used and it was not possible to know whether the sympathomimetic amine was acting directly on the heart or whether the latter was being largely influenced by secondary factors, e.g. reflex autonomic nerve activity, increased plasma potassium and free fatty acids etc.

Consequently it appeared desirable to investigate the arrhythmic effect of the sympathomimetic amines directly on the isolated Ringer-perfused heart (Langendorff preparation) and to measure on a quantitative basis the change produced on a selected parameter which could account for the induction of the arrhythmia. The parameter chosen for study was the ventricular fibrillation threshold (VFT), i.e. the minimal value of a single pulse of current applied during the vulnerable period of late systole which was required to induce ventricular fibrillation or a rapid tachycardia in the isolated perfused heart of the rabbit; Han, de Jalon & Moe (1964) had shown in the dog anaesthetized with pentobarbitone that an intravenous infusion of adrenaline or noradrenaline initially lowered the VFT.

The purpose of the present investigation was four-fold in its approach. (1) to compare the effect of four catecholamines (adrenaline, noradrenaline, isoprenaline and dopamine) and two other sympathomimetic amines on the VFT in the rabbit isolated perfused heart, (2) to demonstrate whether this effect was mediated via α- or β-adrenoceptors, (3) to determine whether chloroform sensitizes the ventricular muscle to catecholamines by potentiating the lowering effect of the latter on the VFT, and (4) to study whether carbachol, used to simulate increased parasympathetic activity, influences the effect of adrenaline on the VFT. The results indicate that the catecholamines, with the exception of dopamine, significantly lower the VFT but that the α-agonists phenylephrine and methoxamine are ineffective, that this lowering of the VFT is abolished by β-adrenoceptor blockade but not by α-blockade, that adrenaline, but not isoprenaline, lowers the VFT significantly more in the presence of chloroform; and that the lowering effect of adrenaline on the VFT is not significantly increased by carbachol.

Methods

Ventricular fibrillation or a rapid tachycardia was induced in rabbit hearts perfused via the aorta with McEwen's (1956) solution at 37°C by the method described previously (MacConaill & Murnaghan, 1967; Murnaghan, 1971; 1973). The fibrillation threshold was determined by measuring the minimal strength needed to produce arrhythmia of a 10 ms square-wave pulse of current applied after each 8th heart beat during the vulnerable period of late systole. It was achieved by testing at 5 ms steps during the latter half of the R-T interval. The minimal acceptable duration of the induced arrhythmia was set at 1 s because of difficulty in differentiating it from groups of 3 or 4 multiple extrasystoles.

The type of arrythmia induced, monitored on a large oscilloscope screen (Airmec), was recorded as fibrillation or tachycardia. The duration of the arrhythmia was listed as persistent (> 60 s) or non-persistent. If a normal rhythm had not returned after 60 s, defibrillation was effected by manually infusing 0.3 M KCl (usually 0.5 ml sufficed) into the aortic cannula. The term ventricular fibrillation threshold (VFT) was used to indicate the minimal current required to induce either fibrillation or tachycardia. In order to indicate the magnitude of change produced by the sympathomimetic amine on the VFT despite the variation in magnitude of the latter among hearts, the VFT change ratio was calculated, i.e. the VFT in the presence of the sympathomimetic amine/VFT of the control. If the sympathomimetic amine was combined with another drug then the latter alone acted as control. The minimal time after the R wave of the ECG at which the 10 ms pulse had to be applied to induce the arrhythmia was called the vulnerable time.

The perfusion apparatus consisted of three heat-exchange glass columns connected to a perspex block fitted with a tap so that the fluid from any one column could be selected. Each column was fed from a reservoir bottle, the perfusion fluid was gassed with 5% CO_2 in O_2 within each column. At the front of the block an opening, with tap, permitted the injection of KCl into the perfusion fluid just above the aortic cannula, at the back of the block was an opening with tap for removal of air bubbles from the cannula when required. All drugs with the exception of the catecholamines were incorporated in the perfusion fluid. To avoid oxidative destruction of the catecholamine it was dissolved in 160 mM sodium chloride acidified with HCl to pH 5 and infused by a syringe pump at a constant rate into the aortic cannula via an opening on the side of the perspex block. Infusion of the acidic saline solution alone failed to influence the VFT. While the infusion rates for a catecholamine were very similar in the different hearts, its concentration in the perfusion fluid unavoidably varied because of the variable coronary perfusion flow rates. However, the mean concentrations did not differ significantly between the groups under comparison.

The results are usually expressed by the arithmetic mean with its standard error. Significance of difference was determined by Student's *t* test or the Chi-square test where appropriate.

Drugs used were: adrenaline hydrogen tartrate (Farbwerke Hoechst AG.), noradrenaline bitartrate (Winthrop), isoprenaline hydrochloride (Winthrop), dopamine hydrochloride (Calbiochem.), phenylephrine hydrochloride (Winthrop), methoxamine hydrochloride (Burroughs Wellcome & Co.), propranolol (ICI), pindolol (Sandoz Products Ltd.), phentolamine (Ciba), carbachol (Koch-Light) and chloroform (B.D.H.).

Results

All four catecholamines adrenaline, noradrenaline, isoprenaline and dopamine when infused for 2-15 min at rates indicated in Table 1 caused in most trials an increase in the diastolic tension of the heart, a positive inotropic and chronotropic response and an increase in coronary flow of approximately similar magnitudes. This increase in coronary flow induced by the catecholamine largely disappeared after several hours of perfusion by which time the heart had become oedematous. Propranolol 0.3 μM and pindolol 1 μM completely inhibited the chronotropic but not the inotropic response of the catecholamine.

Phenylephrine and methoxamine, which are considered to be 5-10 times less potent than adrenaline as pressor agents, were added to the perfusion fluid to give a concentration which was an equivalent number of times greater than that attained by adrenaline during its infusion. Both drugs increased the diastolic tension but had little or no effect on the systolic force or coronary flow; phenylephrine, however, increased the heart rate in three out of five trials.

In two experiments the Q-T interval was measured during the infusion of adrenaline. It shortened more than could be accounted for by the increase in heart rate suggesting a direct shortening in the duration of the ventricular action potential.

Effect of sympathomimetic amines on the ventricular fibrillation threshold

Adrenaline when infused at a rate of 8-32 nmol/min lowered the VFT in 22 out of 24 trials. The mean infusion rate of 24 ± 2.5 nmol/min corresponded to a concentration of 2.44 ± 0.44 μM in the perfusion fluid and the mean duration of the infusion was 6 ± 0.58 minutes. This lowering of the VFT could be produced at least 2-3 times in the same heart and had passed off 15-30 min after termination of the infusion. The preceding control values for the VFT varied between 3.2-20 mA in the 24 trials on 11 rabbit hearts; the range during the adrenaline infusion was 0.9-9 mA. The mean VFT change ratio (VFT of test/VFT of control) of 0.56 ± 0.05 was significantly different from unity at the 0.1% level (Table 1). It did not differ significantly among the three subgroups at infusion rates of 8, 16 and 32 nmol/minute. Noradrenaline at a mean infusion rate of 23.3 ± 2.5 nmol/min (concentration 2.15 ± 0.35 μM) lowered the VFT in 8 out of 11 trials on 3 hearts while isoprenaline at approximately one-fifth the infusion rate (and concentration) of the other two lowered it in 7 out of 12 trials in 6 hearts. Their respective VFT change ratios of 0.72 ± 0.08 and 0.79 ± 0.06 were significantly different from unity but not from each other. However the value for isoprenaline, but not of noradrenaline, was significantly different ($P < 0.01$) from that for adrenaline. Both dopamine, when infused at a rate approximately 100 times greater than that of adrenaline, and phenylephrine and methoxamine, when perfused in a concentration approximately 9 times greater than that attained by adrenaline failed to alter significantly the VFT change ratio (Table 1).

In order to determine whether the sensitization produced by chloroform of the ventricle to

Table 1 Effect of sympathomimetic amines on induced ventricular fibrillation

Amines	*Drug*	*Infusion rate (nmol/min)*	*Concentration (μM) Drug or Amine*	*Proportion of trials VFT was reduced*	*VFT change ratio (Mean ± s.e. mean)*	*Value P*
Adrenaline	–	24 ± 2.5	–	22/24	0.56 ± 0.05	0.001
Adrenaline	Chloroform	32	1 mM	8/8	0.24 ± 0.01	0.001*
Adrenaline	Carbachol	28 ± 4.0	0.4	4/4	0.60 ± 0.05	NS*
Adrenaline	Phentolamine	32	1.0	3/3	0.49 ± 0.17	NS*
Adrenaline	Beta-blockade	32	P – 0.3 L – 1.0	0/2	1.14 ± 0.15	0.01*
Noradrenaline	–	23 ± 2.5	–	8/11	0.72 ± 0.08	0.02
Noradrenaline	Propranolol	16	0.3	0/1	1.22	NS*
Isoprenaline	–	5.1 ± 1.2	–	7/12	0.79 ± 0.06	0.01
Isoprenaline	Chloroform	3.7 ± 1.2	1 mM	3/6	0.82 ± 0.18	NS*
Dopamine	–	2460 ± 530	–	3/8	0.95 ± 0.06	NS
Phenylephrine	–	–	23.2 ± 4.8	2/5	0.97 ± 0.15	NS
Methoxamine	–	–	20.0 ± 5.8	1/4	1.26 ± 0.32	NS

* Comparison with catecholamine alone in same hearts.
P—propranolol, L—pindolol, in column 4.

develop arrhythmias and even ventricular fibrillation when exposed to adrenaline could be related to an increased lowering of the VFT, chloroform was incorporated in the McEwen's solution in a concentration of 1 mM which is approximately equivalent to the blood concentration achieved during anaesthesia with this agent. The chloroform caused a temporary depression in cardiac contractility and an insignificant increase in the VFT. When adrenaline was now infused the VFT was markedly reduced; the mean VFT change ratio of 0.24 ± 0.01 in 8 trials on three hearts was significantly lower ($P < 0.001$) than that produced by adrenaline in the absence of chloroform (0.53 ± 0.05) in a similar number of trials on the same hearts (Table 1). When, however, isoprenaline was infused in the presence of chloroform it failed to lower the VFT more than that of isoprenaline alone. This result confirms the work of Garb & Chenoweth (1948) who showed that chloroform sensitized the ventricles of the cat *in situ* to fibrillate to adrenaline but not to isoprenaline.

In order to determine whether parasympathetic activity could increase the predisposition to ventricular fibrillation produced by adrenaline, carbachol was incorporated in the McEwen's solution in a concentration of 0.25-1 μM. During the perfusion with carbachol the heart rate fell so markedly that the atria had to be driven by a second stimulator at a rate of 60-80% that of the control spontaneous rate. More rapid driving could not be used as it resulted in a 2/1 A-V block. The carbachol alone did not significantly alter the VFT and when adrenaline was now infused it caused a reduction in the mean VFT change ratio to 0.60 ± 0.05 which was not significantly different from that with adrenaline alone (Table 1).

In order to determine whether the lowering effect by the catecholamines on the VFT was mediated via α- or β-adrenoceptors, the α-adrenoceptor blocking agent phentolamine was added to the perfusion fluid to give a concentration of 1 μM. Phentolamine had no effect on the VFT and it did not prevent adrenaline from lowering it (Table 1) indicating that the reduction of the VFT produced by adrenaline is not exerted via the α-receptors. This was confirmed by the finding that the α-agonists phenylephrine and methoxamine failed to lower the VFT. When propranolol (0.3 μM) or pindolol (1 μM) was included in the perfusion fluid they prevented adrenaline or noradrenaline from lowering the VFT, indicating that the reduction of the VFT by the catecholamine is exerted via the β-adrenoceptors. In the concentrations used the two β-adrenoceptor blocking agents alone did not alter the VFT.

Effect of the sympathomimetic amines on the duration of the arrhythmia

In order to determine whether the sympathomimetic amines influenced the duration of the arrhythmia, the proportion of trials in which the induced arrhythmia was persistent was recorded. In 98 out of 163 trials the arrhythmia was persistent in the control and in 22 out of 30 trials during the test in those hearts where adrenaline was infused. The respective values for the control and test with the other sympathomimetic amines were: noradrenaline 26/42 and 10/12, isoprenaline 58/103 and 13/13, dopamine 25/41 and 6/8, phenylephrine 24/41 and 11/11 and methoxamine 20/31 and 10/15. Although adrenaline, noradrenaline, isoprenaline and dopamine increased the proportion of persistent arrhythmias when com-

Table 2 Effect of sympathomimetic amines on vulnerable times in rabbit isolated heart

	Control (ms)	*Test (ms)*	P *value*
Adrenaline	82 ± 1.7 (175)	71 ± 5.1 (30)	< 0.02
Adrenaline + carbachol	94 ± 4.2 (17)	60 ± 18.6 (4)	NS
Adrenaline + chloroform	111 ± 3.0 (10)	63 ± 13.8 (4)	< 0.001
Adrenaline + β-blockade	66 ± 7.5 (6)	90 ± 5.8 (3)	NS
Adrenaline + phentolamine	106 ± 3.7 (16)	43 ± 14.4 (4)	< 0.001
Noradrenaline	62 ± 2.1 (46)	50 ± 5.3 (12)	< 0.05
Noradrenaline + β-blockade	60 ± 5.8 (3)	45	NS
Isoprenaline	84 ± 1.6 (121)	61 ± 5.8 (12)	< 0.001
Isoprenaline + chloroform	96 ± 3.9 (16)	74 ± 17.6 (6)	NS
Dopamine	77 ± 3.1 (37)	78 ± 11.2 (6)	NS
Phenylephrine	63 ± 3.0 (49)	62 ± 4.6 (11)	NS
Methoxamine	75 ± 1.9 (31)	74 ± 5.0 (16)	NS

Mean value with s.e. mean in ms is given. Number of values indicated in parentheses.

pared with the corresponding controls, the increase was not significant except for isoprenaline ($P < 0.01$) or for all four catecholamines combined ($P < 0.01$). Of the remaining two sympathomimetic amines only phenylephrine significantly increased ($P < 0.05$) the proportion of arrhythmias which were persistent.

Chloroform and carbachol alone did not alter the proportion of the arrhythmias which were persistent. During the adrenaline infusion the arrhythmia was persistent whether chloroform or carbachol was or was not present. There was no indication that the drugs used altered the type (fibrillation or tachycardia) of the induced arrhythmia.

Effect on the vulnerable time

The vulnerable times (VT), i.e. the minimal times in ms after the R-wave at which the electrical square-wave 10 ms pulse was effective in inducing the arrhythmia, are listed in Table 2. Adrenaline alone or when combined with chloroform or phentolamine and noradrenaline and isoprenaline alone significantly shortened the VT. Beta-blockade by propranolol and pindolol prevented this. Although the mean values of adrenaline with carbachol and isoprenaline with chloroform were less than that due to carbachol and chloroform alone respectively, the differences were insignificant because of occasionally large or small values in the former groups. Chloroform and phentolamine alone significantly lengthened the VT from the control McEwen solution values. Beta-blockade and carbachol alone had no significant effect.

Since the vulnerable period occurs during the rapid repolarization phase these results indicate that adrenaline, noradrenaline and isoprenaline, but not dopamine, phenylephrine or methoxamine, either cause the vulnerable period to start at an earlier stage of repolarization or shorten the duration of the ventricular action potential. As adrenaline has been shown to shorten the duration of the Q-T interval the latter is the more probable. Shortening of the duration of electrical systole by these three catecholamines appears to be a prerequisite for lowering of the VFT to occur.

Discussion

The catecholamines adrenaline, noradrenaline and isoprenaline, but not dopamine, when infused at rates which produced similar moderate positive chronotropic and inotropic effects have been shown in this study to lower significantly the ventricular fibrillation threshold (VFT). In a previous preliminary study (MacConaill & Murnaghan, 1967) with adrenaline the results were equivocal. When the adrenaline was infused at the rate of 8 nmol/min, in 8 tests the mean VFT was just significantly lower than the control. However, with infusions of 32 nmol/min a fall in the VFT occurred in three experiments, no change occurred in one and there was a marked rise in the threshold to 5-10 times the control on three separate trials in one experiment only. In the present study where an infusion rate of adrenaline of 8-32 nmol/min was used the VFT was lowered in 22 out of 24 trials and a rise in threshold was never seen. At least one essential difference existed between the two studies. In the initial study the average time after the start of the adrenaline infusion at which the threshold was determined was approximately 2.5 min; in this study it was 6 minutes. These results would appear to suggest that the lowering effect on the VFT may be more apparent after a sufficient elapse of time. This fall in the VFT is clearly mediated via β-adrenoceptors because it is blocked by β- but not α-receptor blocking agents. Although Papp & Szekeres (1968a) had previously demonstrated this in the whole anaesthetized animal the present study clearly shows that the action can be exerted directly on the heart itself. It is presumed that this fall induced in the VFT during the administration of adrenaline, noradrenaline and isoprenaline predisposes to the initiation of the rapid ventricular tachycardia or fibrillation; whether it contributes to the maintenance of the arrhythmia remains equivocal. Although all the catecholamines increased the incidence of a persistent arrhythmia this change was only statistically significant with isoprenaline.

Han *et al.* (1964) showed that in the pentobarbitone anaesthetized dog an intravenous infusion of adrenaline or noradrenaline first lowered the VFT of the heart *in situ* and subsequently raised it but that left stellate ganglion stimulation only lowered it. Papp & Szekeres (1968a) in an extension of this study showed that isoprenaline only lowered the VFT in the dog. These results in conjunction with those in this present study suggest that the rise in the VFT is possibly extracardiac in origin and may involve excitation of α-adrenoceptors.

It is believed that ventricular fibrillation is usually triggered by an ectopic beat falling on the early part of the T-wave of the preceding beat. During this vulnerable period there is a lack of homogeneity in recovery of excitability of the ventricular fibres so that aberrant rhythms can arise if a sufficient flow of current occurs between fibres in different stages of polarization. Such a flow can be triggered by an ectopic beat or by an externally applied pulse of current when it occurs during this vulnerable phase of the cardiac cycle. If this lack of homogeneity is magnified it is possible that the arrhythmia can originate spontaneously because the endogenous current generated will now be adequate to initiate it. Such a phenomenon is referred to as microre-entry. Adrenaline initially not only shortens the refractory period of ventricular muscle but also exaggerates the inhomogeneity as measured by the increase in temporal dispersion of the refractory period, subsequently it lengthens the refractory period and decreases its temporal dispersion in the whole animal (Papp & Szekeres, 1968b). These respective early and late changes appear to correlate well with the initial decrease and subsequent increase in the VFT.

Dopamine is a catecholamine which is being used increasingly to treat patients with congestive heart failure, shock and hypotension. It is of interest to note in this study that when used in an approximate equipressor concentration to that of noradrenaline it failed to lower the VFT. Katz, Lord & Eakins (1967) have recommended its use in hypotension and shock in patients anaesthetized with cyclopropane or halogenated hydrocarbons because it appeared less likely to induce cardiac arrhythmias than the other sympathomimetic amines which are used as vasoconstrictor agents.

Phenylephrine and methoxamine have also been advocated as the pressor agents of choice particularly during anaesthesia with cyclopropane and halogenated hydrocarbons (Orth, Leigh, Mellish & Stutzman, 1939, Stutzman, Pettinga & Fruggiero, 1949) as they were less likely to predispose to ventricular arrhythmias than the other sympathomimetic amines tested. This belief is confirmed by the evidence in this study in that both amines failed to lower the VFT. In fact Papp & Szekeres (1968a) showed in the anaesthetized dog that phenylephrine and methoxamine raised the VFT but as explained previously, this increase may be due to an indirect action as it did not occur here in the isolated heart. Although the results of this study suggest that methoxamine may be preferable to phenylephrine because it did not increase the proportion of persistent arrhythmias, it is improbable that this finding is of significance in man because ventricular fibrillation is invariably persistent in the human species.

The results of this and the previous study by the author clearly indicate that chloroform potentiates the lowering effect of adrenaline on the VFT. It appears logical to assume that this effect might play an important role in the production of ventricular fibrillation by adrenaline during sensitization with chloroform and other substituted and unsubstituted hydrocarbons. It is of interest to note that Garb & Chenoweth (1948) failed to induce ventricular fibrillation when isoprenaline was injected into the cat anaesthetized with chloroform. This would appear to substantiate the finding in this study that chloroform failed to increase the lowering effect of isoprenaline on the VFT. However, other factors apart from sensitization could account for this fortuitous agreement. These workers used a very large dose of isoprenaline which would have caused considerable hypotension due to its vasodilator property. This effect may be of significance because hypertension induced by a sympathomimetic amine may contribute, in part, towards the induction of an arrhythmia (Nickerson & Nomaguchi, 1949, Nickerson & Smith, 1949). Katz (1965) showed that chloroform sensitized the heart to a non-malignant arrhythmia when he used threshold doses of isoprenaline. It would appear that the sites of origin of tachycardias and ventricular fibrillation may be different (Dresel & Sutter, 1961). Nathanson & Miller (1949) have presented evidence to indicate that multifocal arrhythmias including fibrillation induced by adrenaline originate in ventricular and/or Purkinje muscle while those arrhythmias induced by isoprenaline arise invariably at the A-V node. Indeed Dresel & Sutter (1961) could only induce a nodal rhythm in the dog anaesthetized with cyclopropane despite the use of very large doses of isoprenaline.

The results on the effect of carbachol are equivocal. In four trials in the previous study by the author carbachol significantly ($P < 0.01$) potentiated the effect of adrenaline; in four trials in the present study it failed to do so. When the results are combined the effect is barely significant at the 5% level. As mentioned earlier, adrenaline alone in the previous study only lowered the VFT slightly but exerted a considerable lowering effect in this study. This suggests that carbachol can only potentiate adrenaline if the effect of adrenaline alone is marginal. It is difficult to compare these results with those in the literature where the experimental conditions have been completely different. It is highly unlikely that an infusion of a parasympathomimetic agent will have identical effects to vagal stimulation because the former could act at sites not reached by the acetylcholine liberated at the vagal nerve terminals. (Compare the effects of an infusion of noradrenaline with stimulation of the sympathetic nerve fibres to the heart on the VFT). Furthermore Dresel & Sutter (1961) have shown that the roles of the vagus in the initiation of ventricular arrhythmias and fibrillation are probably not identical because vagal stimulation in the cyclopropane anaesthetized dog increased the dose of adrenaline required to produce a bigeminy rhythm but not that required to cause ventricular fibrillation.

Apparently the effects of cholinergic activity on sensitization to adrenaline can be as contradictory in the isolated heart as on the heart *in situ*.

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THE ACTION OF NITROGLYCERINE AND DIPYRIDAMOLE IN NORMAL AND ISCHAEMIC DOG HEART

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1 Network and post-network resistances were measured together with heat clearance and heat production in the myocardium.

2 Nitroglycerine dilated all resistance vessels but was twice as effective on network as compared with post-network vessels.

3 Dipyridamole dilated network and post-network vessels equally.

4 Nitroglycerine had no effect on heat clearance or heat production in ischaemic myocardium.

5 Dipyridamole increased heat clearance in normal but not in ischaemic myocardium.

6 Dipyridamole depressed heat production in normal and ischaemic myocardium.

7 That the clinical effectiveness of nitroglycerine does not depend entirely on its coronary vasodilator properties is confirmed.

8 It is suggested that the action of dipyridamole in depressing myocardial heat production may be due to an action on the efficiency of oxygen utilization during cardiac contraction.

Introduction

How vasodilator agents relieve angina is still not understood. Nitroglycerine and dipyridamole offer a nearly classic paradox underlining the problem. Nitroglycerine, although only a moderate coronary vasodilator, remains the most effective clinical preparation in the treatment of angina. Dipyridamole, a much more powerful coronary vasodilator, is relatively ineffective.

According to Fam & McGregor (1968) nitroglycerine has a prolonged vasodilator action on large conducting coronary arteries but little action on the microcirculation. The ischaemic process itself was thought to bring about local vasodilatation of the microvessels. These workers also showed that dipyridamole had little action on the conducting arteries but was markedly vasodilator to the microcirculation. Assuming the vessels of an ischaemic region to be already fully dilated by hypoxia, dipyridamole can only dilate vessels of the surrounding unaffected myocardium. With no change in calibre of the conducting arteries Fam & McGregor deduced that flow in the ischaemic area 'would be unaffected or even reduced' by dipyridamole. Later the 'steal' phenomon was more specifically invoked; it was concluded that dilatation of vessels surrounding an ischaemic area would reduce perfusion pressure so that the ischaemic area, with its widely dilated blood vessels, would in fact suffer a reduction in blood flow (Rowe, 1970).

There has been recent evidence, however, throwing doubt on the assumption that following acute artery ligation blood vessels in the developing infarct are fully dilated. Indeed there is good evidence of precapillary closure (Grayson, 1973). This would cast doubt on the 'steal' phenomenon as a factor in the clinical ineffectiveness of dipyridamole. Thus there is a clear necessity to re-examine the haemodynamic consequences of acute artery ligation and the actions of both nitroglycerine and dipyridamole on coronary vascular resistance, local tissue blood flow, and heat production in the normal and ischaemic heart.

Methods

Twenty mongrel dogs of either sex, 10 to 16 kg in weight, were anaesthetized with an intravenous injection of sodium pentobarbitone (30 mg/kg). Aortic pressure was measured by means of a cannula positioned in the arch of the aorta via the left common carotid artery and connected to a Statham strain gauge (model P23H). Central venous pressure was measured from a cannula positioned in the right atrium via the right external jugular vein and connected to a Statham strain gauge (model P23AA). Intraventricular pressure was measured with a cannula inserted in the left ventricle through the aortic valves, and connected to a Statham strain gauge (model P23AA). A femoral artery for arterial blood sampling, and a femoral vein for intravenous infusions were also cannulated. Positive pressure ventilation was maintained through a tracheal cannula by means of a Harvard respirator, using a mixture of room air and 95% O_2 and 5% CO_2. The air intake was bubbled through a flask of heated water so that saturated air at an appropriate temperature was delivered to the dog. Arterial blood samples were taken at 15 min intervals throughout the experiment, and arterial pH, $P\text{O}_2$ and $P\text{CO}_2$ were evaluated with a Radiometer Blood Micro System (Type BMS 3). Arterial $P\text{O}_2$ was maintained at 100 mmHg by adjusting the richness of the inhalation mixture, and $P\text{CO}_2$ at 40 mmHg by adjusting the respiration rate. Rectal temperature was monitored with an Elektrolaboratoriet electric universal thermometer (Type T3). Electrocardiogram readings from standard lead II were constantly monitored.

A thoracotomy was performed through the fifth left intercostal space, the pericardium was incised, and the heart suspended in a pericardial cradle. The left anterior descending coronary artery was dissected free as near the origin as possible, and fitted with an electromagnetic flowmeter probe (Zapeda). A snare was placed around the artery distal to the flowmeter probe to obtain zero flow for the calibration of the flowmeter. The anterior descending coronary artery was also dissected free just proximal to the last bifurcation and a ligature placed loosely around it for the purpose of subsequent ligation. Two heated thermocouple probes were used for the assessment of the apparent myocardial thermal conductivity increment over non-perfused tissue (which is directly proportional to local tissue blood flow), and heat production by means of a heat clearance technique (Grayson, Coulson & Winchester, 1971). One probe was implanted in the tissue immediately distal to the occluding ligature, and the other in the tissue proximal to the ligature.

The chest was closed with clamps, and a period of 30 min was allowed for a steady state to be attained. Control measurements were then carried out. Data were recorded continuously on a Varian Statos III electrostatic recorder.

Ten dogs then received an intravenous infusion of nitroglycerine (0.6 mg dissolved in 10 ml isotonic saline) infused over a period of one minute. Ten dogs received a similar infusion of dipyridamole (0.5 mg/kg in 10 ml saline). Data were recorded continuously after administration of either drug until haemodynamic indices returned to control values (usually withon 10 to 12 min for nitroglycerin, and 20 to 25 min for dipyridamole).

The chest was reopened and the distal portion of the anterior descending coronary artery was ligated and cannulated distally for the measurement of peripheral coronary pressure (p.c.p.). The chest was then closed in layers, and a period of 30 min allowed for the achievement of a steady state. An infusion of either nitroglycerine or dipyridamole was then administered hourly for 6 h beginning 1 h after ligation.

Data obtained immediately before the administration of either drug provided control values. Control values for each index represent the mean of a 6-7 min period.

Myocardial vascular resistance was calculated as the ratio of effective perfusion pressure and flow and was calculated for both systole and diastole using peak systolic and end-diastolic parameters. Total coronary vascular resistance was calculated as follows:

$$\frac{\text{aortic pressure} - \text{central venous pressure}}{\text{anterior descending flow}},$$

Total resistance was further partitioned into two components, network resistance and post-network resistance using peripheral coronary pressure. Network resistance was calculated as follows:

$$\frac{\text{aortic pressure} - \text{p.c.p.}}{\text{anterior descending flow}}$$

and represents resistance to flow in vessels of the network, including the large conducting arteries. Post-network resistance was calculated as the difference between total and network resistance, and probably represents the resistance of small arterioles, precapillaries and capillaries. The statistical significance of the data was determined by the paired t-test.

Results

The effect of vasodilator stimuli before and after occlusion of the anterior descending coronary artery

Nitroglycerine. Figure 1 is a record of haemodynamic events from a typical experiment. In the doses used in these experiments, nitroglycerine reduced both systolic and diastolic aortic pressures. Blood flow in the anterior descending coronary artery initially rose both in diastole and in systole. The systolic rise, however, was maximal

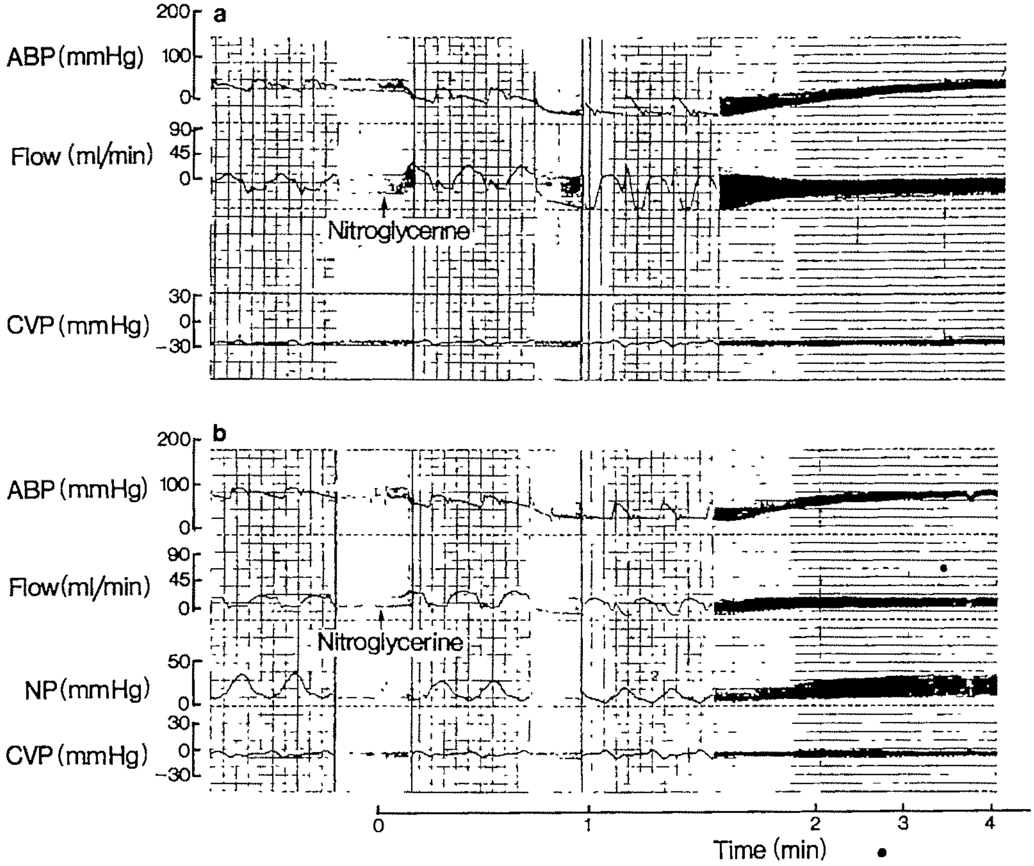

Figure 1 Effect of nitroglycerine on arterial blood pressure (ABP), anterior descending coronary blood flow, central venous pressure (CVP) and peripheral coronary pressure (NP) before and after ligation of the anterior descending coronary artery.

in 15 s and systolic flow began to fall either at the same time as the beginning of the systemic blood pressure drop or shortly after. Diastolic flow continued to rise to maximum levels usually seen at about 20 seconds. Systolic flow fell in most experiments to levels below the preinjection control values. In many experiments it fell briefly to zero and in some experiments as in Figure 1 (those in which the arterial blood pressure fell very markedly), brief periods of backflow were recorded. The total effects were short-lived and usually over within 2 min of the injection.

The effect of nitroglycerine on total resistance before artery ligation, 1 h post-ligation, and 6 h post-ligation is shown in Table 1. Although resistance was increased following ligation, the pattern of change following nitroglycerine administration was not altered. Thus in diastole, resistance at all times, even 6 h after ligation, fell by nearly 30% within 15 s and reached minimum levels of 46% below control levels in 60 seconds. In systole the pattern was different; 15 s after injection (before aortic pressure had fallen) there was a drop of about 30% in resistance but this was not sustained. In some cases there followed brief periods of increased resistance and even zero flow was recorded in some experiments. However, the secondary systolic rise in total resistance was very variable, and in the whole series was not statistically significant.

Table 2 gives the results obtained 1 h post-ligation in terms of network and post-network resistances. At 15 s there was a decline in both network and post-network resistance, similar in systole and diastole. The decline was significant in all cases ($P < 0.05$). At 60 s, the effect of nitroglycerine in diastole was twice as great on network vessels as on post-network vessels. In systole, the effect on resistance was the same in network and post-network vessels, namely an increase which was significant ($P < 0.001$).

Dipyridamole Figure 2 is a record of haemodynamic events from a typical experiment. There was a slow fall in systemic arterial pressure, relatively minor in systole (16 mmHg) but with a more pronounced fall in diastole (32 mmHg).

There was a slowly developing but ultimately very marked increase in diastolic flow in the anterior descending coronary artery, systolic flow showing little change.

After ligation, the picture was essentially the same. The aortic pressure responses were unaltered. Network pressure fell by about 3 mmHg in systole and about 5 mmHg in diastole

Calculated resistances are shown in Table 1. The proportionate changes in resistance following dipyridamole were much the same as before ligation both at 1 h and at 6 hours. Table 2 shows that the maximum effect was much the same on post-network as on network vessels both in systole and in dimstole. Dipyridamole caused about a 50% decline in total resistance with an effect on the network vessels which is greater in diastole than in systole. Its effect on post-network vessels was not significantly greater in diastole.

Effect of nitroglycerine and dipyridamole on myocardial heat production

Figure 3 shows the effect of nitroglycerine and dipyridamole on myocardial heat production expressed as I^2 equivalents.

Nitroglycerine Heat production was not significantly altered by nitroglycerine in either the proximal or the distal myocardium, before or after artery ligation (Figure 3a).

Dipyridamole Myocardial heat production was considerably depressed by dipyridamole (Figure 3b). Before ligation, in both areas of the myocardium, the decline was rapid, minimum levels being reached within 2 minutes. This was followed by a gradual return to control levels.

In the proximal myocardium, dipyridamole

Table 1 Effect of nitroglycerine and dipyridamole on total systolic and diastolic resistance to flow in the territory of supply of the anterior descending artery before and after acute ligation

		Nitroglycerine (n = 10)			*Dipyridamole (n = 6)*	
		Control	*15 s*	*60 s*	*Control*	*max. effect*
Pre-ligation	systole	3.57 ± 0.53	2.86 ± 0.32	3.97 ± 0.64	6.65 ± 1.51	3.02 ± 0.73
	diastole	1.84 ± 0.24	1.10 ± 0.12	1.02 ± 0.10	2.43 ± 0.51	1.15 ± 0.36
1 h post-ligation	systole	6.00 ± 1.40	4.60 ± 1.10	9.60 ± 3.40	6.40 ± 1.95	2.76 ± 0.64
	diastole	3.30 ± 0.70	2.30 ± 0.50	1.90 ± 0.40	2.11 ± 0.59	0.76 ± 0.19
6h post-ligation	systole	4.90 ± 1.13	4.00 ± 1.10	4.80 ± 1.50	5.90 ± 1.76	2.90 ± 0.70
	diastole	2.40 ± 0.60	1.70 ± 0.50	1.10 ± 0.20	2.37 ± 0.60	1.20 ± 0.30

Resistance is expressed in peripheral resistance units.
Values are mean ± s.e. mean.

Table 2 The effect of nitroglycerine and dipyridamole on network and post-network resistances one hour after ligation.

		Network		*Post-Network*	
		Systolic	*Diastolic*	*Systolic*	*Diastolic*
Nitroglycerine	15 s	−20.1 ± 3.4*	−28.6 ± 5.3*	−28.0 ± 8.1*	−22.2 ± 5.4*
($n = 10$)	60 s	+36.5 ± 20.8**	−46.1 ± 5.4	+37.8 ± 2.4**	−22.0 ± 5.0
Dipyridamole ($n = 6$)		−40.7 ± 8.6**	−60.7 ± 7.8**	−48.9 ± 9.4**	−55.0 ± 8.2**

Results are expressed as % change. Nitroglycerine = 0.6 mg; dipyridamole = 0.5 mg/kg
With nitroglycerine at 60 s, the diastolic decline in network resistance was significantly greater than diastolic decline in post-network resistance ($P < 0.001$) With dipyridamole, network decline in diastolic resistance was significantly greater than decline in systolic resistance ($P < 0.05$).
Values are mean ± s.e. mean. *$P < 0.05$; **$P < 0.001$.

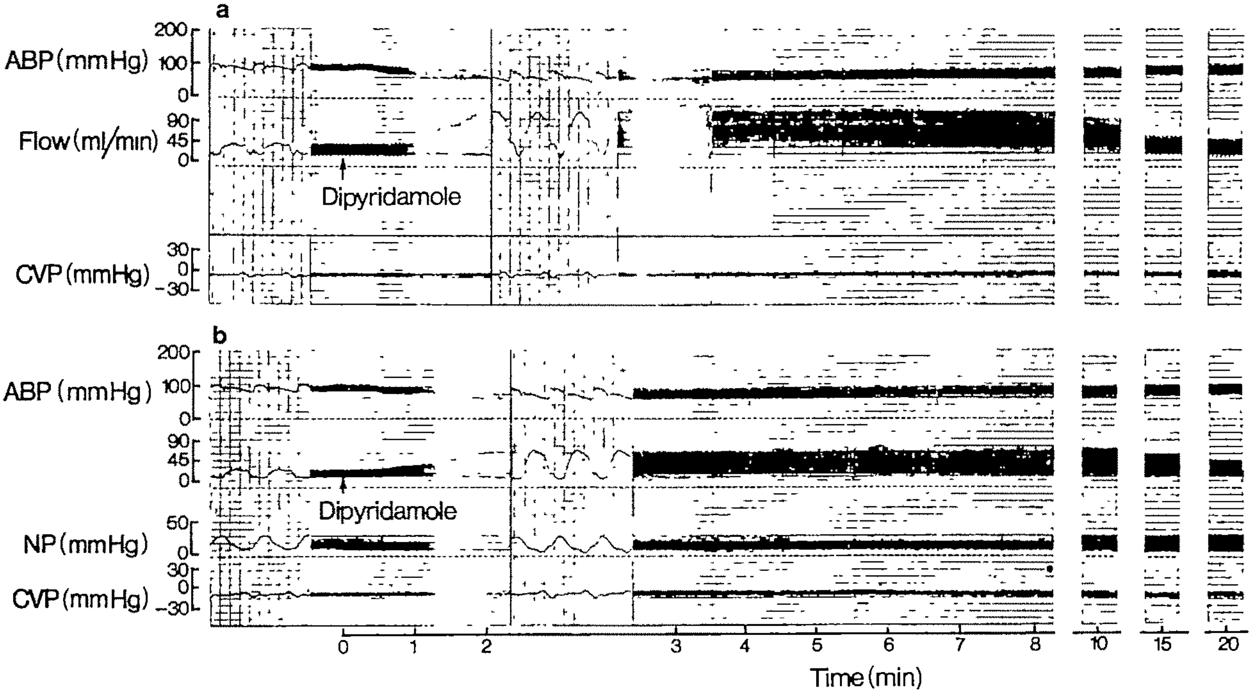

Figure 2 The effect of dipyridamole on arterial pressure (ABP), flow in the anterior descending coronary artery, central venous pressure (CVP) before ligation and its effect on the same parameters together with peripheral coronary pressure (NP), post-ligation.

produced similar declines in heat production throughout the experiment. The decline in heat production before ligation was 65% ($P < 0.05$). At 1 h and at 6 h post-ligation the declines were 69% ($P < 0.01$) and 41% ($P < 0.01$) respectively.

However, in the distal myocardium the effect of dipyridamole was altered after ligation. Before ligation, dipyridamole produced a decline in heat production of 54% ($P < 0.01$). One hour following ligation, the control level of heat production in the ischaemic muscle was depressed, showing some recovery by 6 hours. In spite of the fact that initial levels of heat production were lower following ligation dipyridamole still produced a further decline in heat production, although the magnitude of the response was reduced. Thus 1 h post-ligation the fall in heat production produced by dipyridamole was 39% ($P < 0.05$), and at 6 h the fall was 32% ($P < 0.05$).

The effect of nitroglycerine and dipyridamole on myocardial thermal conductivity increment (Δk).

Figure 4 shows the effect of nitroglycerine and dipyridamole on heat clearance (thermal conductivity increment) expressed in thermal conductivity units.

Nitroglycerine In the proximal myocardium, before ligation, the initial slight rise and subsequent decline in flow following the administration of nitroglycerine (Figure 4a) were not statistically significant. However, at 1 h and at 6 h the flow declines of 27% and 19% respectively were significant ($P < 0.05$).

In the distal myocardium, before ligation, nitroglycerine injection caused a significant decline in flow of 30% ($P < 0.05$). One hour post-ligation the fall was 38% ($P < 0.05$). Six hours post-ligation the decline in flow was not significant.

Dipyridamole Before ligation, dipyridamole had marked effects on local blood flow in both the proximal and the distal myocardium (Figure 4b). Flow increased, reaching maximal levels in about 4 min, thereafter declining gradually toward control values.

In the proximal myocardium, before ligation, dipyridamole increased Δk by 134% ($P < 0.05$). Post-ligation the dilator effect was sustained. At 1 h dipyridamole increased flow by 95%

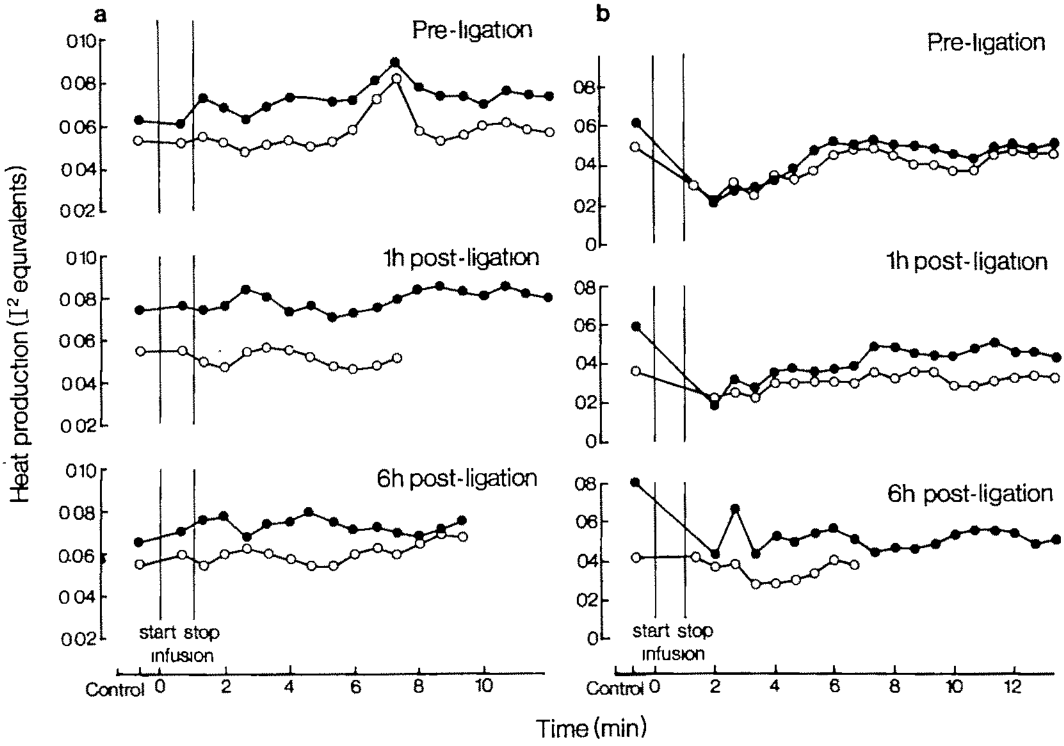

Figure 3 The effect of nitroglycerine (a) and dipyridamole (b) on myocardial heat production (I^2 equivalents) before ligation and 1 and 6 h post-ligation. Proximal myocardium (●); distal myocardium (○).

($P < 0.001$), and at 6 h increased flow by 75% ($P < 0.05$). The 6 h effect was perhaps somewhat minimized by the fact that there was a developing hyperaemia in the area surrounding the zone of ischaemia so that at 6 h control values were 40% higher than pre-ligation control values.

In the distal myocardium prior to ligation, dipyridamole increased local myocardial flow by 74% ($P < 0.05$). After ligation however, the effectiveness of dipyridamole in increasing flow was effectively abolished. At 1 h post-ligation, although Δk increased slightly, this change was not significant. At 6 h no significant change occurred in levels of Δk. Following ligation there was a gradual decline in baseline levels of heat clearance. By 6 h it was 15% below pre-ligation levels.

Discussion

Action of dipyridamole and nitroglycerine on the coronary circulation

Dipyridamole has been described as acting mainly on small resistive arterioles, with little effect on the large conducting arteries of the coronary circulation. Nitroglycerine has been described as acting mainly on large arteries conducting blood towards the microcirculation (Fam & McGregor, 1968). However, in our experiments dipyridamole markedly reduced vascular resistance, having an equal effect on network and post-network vessels, and being equally effective in systole and diastole. The term 'network' describes the subepicardial plexus of vessels larger than 70 μm and includes the main conducting arteries; 'post-network' vessels are the vessels derived from the network and are mainly precapillary and capillary, both superficial and deep. Systolic vascular resistance has a large extravascular component. However, diastolic vascular resistance has been shown to be largely due to blood vessel tone. Our present evidence, therefore, shows that although according to Fam & McGregor (1968) dipyridamole has no dilator action on the large conducting arteries, it does have a pronounced vasodilator action on all other vascular elements of the coronary circulation, dilating network vessels (arteriolar or larger in size) as effectively as post-network vessels. Our evidence also shows that Fam & McGregor

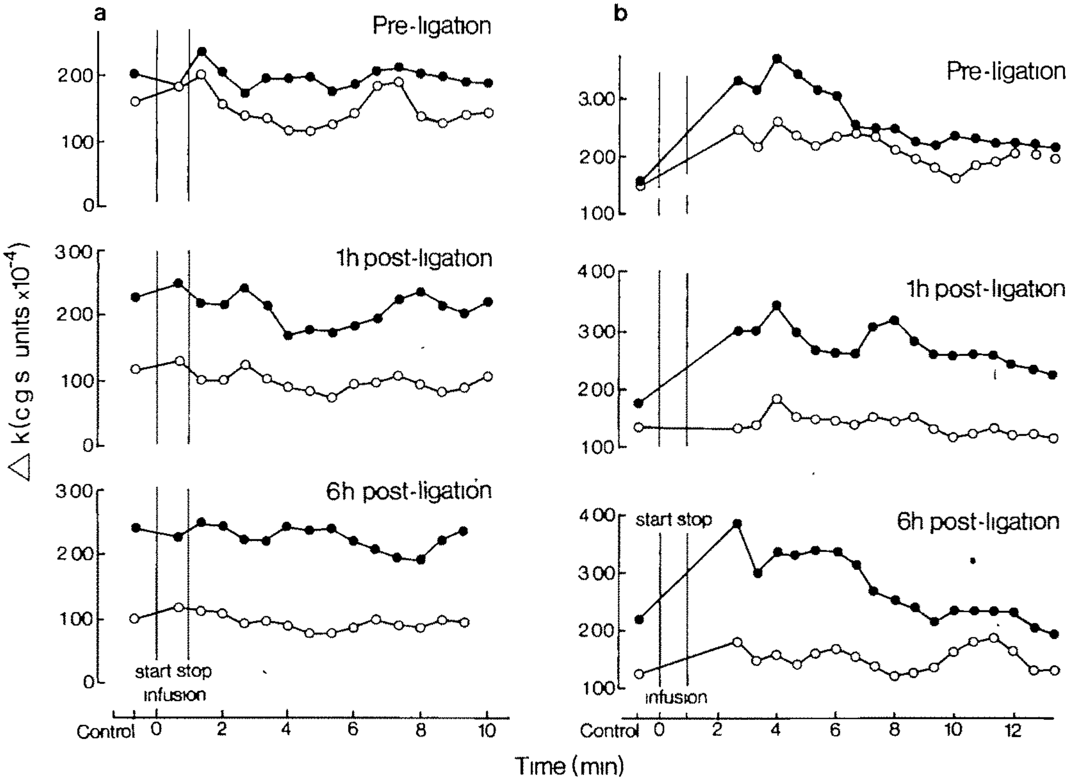

Figure 4 The effect of nitroglycerine (a) and dipyridamole (b) on myocardial heat clearance (Δk) before ligation and 1 and 6 h post-ligation. Proximal myocardium (●); distal myocardium (o).

underestimated the dilator effect of nitroglycerine on the microcirculation. In our experiments nitroglycerine markedly lowered diastolic post-network resistance, showing it to be a marked vasodilator of microcirculatory vessels. However, it was twice as effective in dilating the larger network vessels, which include the conducting arteries referred to by Fam & McGregor. The action of nitroglycerine in systole is more complex. After an initial period of vasodilatation of both network and post-network vessels there followed a variable period of increased resistance. In some cases flow ceased altogether for a very brief period. In general nitroglycerine improved diastolic flow and reduced systolic flow, although heat clearance studies showed little overall effect on tissue blood flow. Since heat production was not significantly affected, it must be assumed that augmented diastolic flow levels compensated for the reduced systolic flow and were sufficient to supply the nutritional needs of the heart. The systolic increase in resistance was probably extravascular arising from increased force of contraction, a baroreceptor response to the 33% fall in blood pressure. The difference between these effects of nitroglycerine and the effects of amyl nitrate which markedly reduced myocardial heat production and increased heat clearance (Grayson, Irvine & Parratt, 1967) should be noted.

The effect of dipyridamole on myocardial heat production

Dipyridamole significantly depressed myocardial heat production in the intact heart. That this is not an artefact due to increased blood flow is shown by the fact that blood flow continued to increase after stabilization of heat production. Moreover in the ischaemic myocardium dipyridamole had no effect on flow, however it still produced significant inhibition of heat production. The metabolic actions of dipyridamole are complex and controversial. There seems to be some agreement however, that dipyridamole prevents the deamination of adenosine (Kübler, Spieckermann & Bretschneider, 1970). It might not be surprising for the net effect of this to be a diminution in the liberation of free energy from adenosine 5′-triphosphate stores. If this were the case, this might be expected to lead to a reduction in myocardial contractility. However, many investigators report that dipyridamole has no inotropic effect (Kiese, Lange & Resag, 1960; West, Bellet, Manzoli & Müller, 1962), whereas others report increased contractility (Elliot, 1961; Yu & Gluckman, 1969). The most probable conclusion therefore is that dipyridamole, directly or indirectly, leads to increased efficiency of utilization of free energy by the contractile mechanisms. This would decrease the amount of energy lost as heat whilst maintaining the initial force of contraction.

The action of nitroglycerine in the ischaemic myocardium

Heated thermocouple measurements showed that nitroglycerine had little effect on heat clearance or heat production in the ischaemic myocardium. This is similar to earlier findings using xenon clearance (Pasyk, Bloor & Gregg, 1968) and adds weight to the suggestion that the clinical effectiveness of this preparation is independent of its vasodilator properties. It has been suggested that the major factor giving rise to angina or infarction is not so much a total obliteration of blood supply to a tissue, but the generation of a relative hypoxia brought about by a vascular inflow into a region which is insufficient for its work load. The present evidence, therefore, is consistent with the suggestion that nitroglycerine may work through its general action on peripheral resistance which leads to a fall in blood pressure and, consequently, a reduced overall cardiac work load.

The action of dipyridamole in the ischaemic myocardium

In the present experiments, dipyridamole did not improve blood flow to the ischaemic myocardium. This is consistent with the findings of other investigators (Pasyk *et al.*, 1968; Schaper, 1971; Benzing, Wahl, Bender & Rabe, 1973). It has already been observed that in the developing infarct there is a severe reduction of capillary blood flow, possibly due to precapillary vasospasm, with most of the remaining flow passing through larger anastomosing vessels (Grayson & Scott, 1973). This would not be compatible with the 'steal' phenomenon (Fam & McGregor, 1968, Rowe, 1970) in which it is assumed that vessels in the ischaemic area are maximally dilated as a result of hypoxia. However, the vascular changes in acute infarction are probably complex. We have shown that distal to an acute ligation, islands of ischaemic tissue alternate with areas which are well perfused. It is probable that in the well perfused tissue blood vessels are maximally dilated. The clinical ineffectiveness of dipyridamole may well be due to the fact that it is not able to reach the appropriate vascular smooth muscle sites in areas of vasospasm, whereas in the other areas which it can reach blood vessels are already maximally dilated by virtue of hypoxia. In these areas, too, it can lower heat production without further action on blood vessels. It should be noted that heat production which, among other things depends on capillary flow, showed considerable improvement during the 6 h observation period whilst pressures and vascular resistances did not change. Pressure gradients were therefore sufficient for blood delivery through anastomotic routes. This strongly favours an active factor in the initial failure of capillary filling.

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THE EFFECT OF VARIOUS AMINE-DEPLETING DRUGS ON THE FEVER RESPONSE EXHIBITED BY RABBITS TO BACTERIAL OR LEUCOCYTE PYROGEN

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1 The concentration of noradrenaline, dopamine and 5-hydroxytryptamine (5-HT) in the rabbit brainstem was measured during fevers produced by either an injection of bacterial pyrogen (BP) or continuous infusion of leucocyte pyrogen (LP).

2 Both procedures had little effect on the concentration of noradrenaline in the preoptic/hypothalamic area but significantly ($P < 0.001$) lowered the concentration of noradrenaline in the midbrain and pons/medulla.

3 BP significantly ($P < 0.01$) lowered the concentration of 5-HT in the preoptic/hypothalamic area but had no effect in the midbrain or pons/medulla, whereas LP significantly ($P < 0.01$) lowered the concentration of 5-HT in the midbrain and pons/medulla but had little effect in the hypothalamus.

4 The concentration of dopamine throughout the brainstem was little affected by either BP or LP fevers. However the concentration in the midbrain was significantly reduced by LP ($P < 0.001$).

5 α-Methyltyrosine (200 mg/kg) pretreatment diminished the pyrogenic response to both BP and LP whilst *p*-chlorophenylalanine (300 mg/kg) slightly enhanced the response to both forms of challenge.

6 Reserpine (1 mg/kg) diminished both types of fever whilst a combination of α-methyltyrosine and *p*-chlorophenylalanine slightly enhanced the fevers produced by either BP or LP.

7 The results obtained are discussed in relation to the mechanisms involved in the production of fever and to the possible function of noradrenaline and 5-HT as thermoregulatory transmitters.

Introduction

It has long been recognized that the intravenous injection of certain substances produce fever both in man and in animals and the term pyrogen has been traditionally used to describe these substances. Although it is now commonly accepted that bacterial (or exogenous) pyrogen fever is ultimately caused by leucocyte (or endogenous) pyrogen (Atkins & Wood, 1955a, b), there is still controversy concerning a site of action for pyrogen. Cooper, Cranston & Honour (1967) injected pyrogens directly into rabbit brain in an attempt to identify the site of action. They concluded that bacterial pyrogen provoked the release of leucocyte pyrogen which in turn acted on the anterior hypothalamus. More recently, Rosendorff & Mooney (1971) produced fever by injecting leucocyte pyrogen into the lower brain stem from which they concluded that there may be more than one site of action.

The theory that pyrogens produce fever indirectly by the release of hypothalamic noradrenaline or 5-HT was first suggested by Feldberg & Myers (1963). Subsequent experiments, designed to test the possible involvement of these amines in the genesis of fever, have provided equivocal results. Thus reserpine has been shown to protect rabbits from bacterial pyrogen fever (Göing, 1959; Kroneberg & Kurbjeweit, 1959; Des Prez, Helman & Oates, 1966) although Cooper *et al.* (1967) found that intraventricular administration of reserpine did not suppress bacterial pyrogen fever. Similarly, Des Prez *et al.* (1966) found that bacterial pyrogen fever was not associated with changes in hypothalamic noradrenaline or 5-HT concentrations whereas Canal & Ornesi (1961), Kuruma, Takagi & Yanada (1964) and Takagi & Kuruma (1966) all found hypothalamic 5-HT stores to be depleted. Giarman, Tanaka, Mooney & Atkins (1968) found that both amines were depleted. Feldberg (1968) has sought to resolve these discrepancies and also the observation that 5-HT injected directly into the CNS lowers rather than raises body temperature in rabbits (Cooper, Cranston & Honour, 1965) by suggesting that the amines may act as thermoregulatory transmitters at additional sites caudal to the hypothalamus.

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The present experiments were undertaken (a) to investigate the possibility that noradrenaline and 5-HT function as thermoregulatory transmitters at sites caudal to the hypothalamus (b) to compare the effects of leucocyte pyrogen and bacterial pyrogen on the cerebral concentration of these amines (c) to use specific amine depleting drugs to investigate the fever suppressing properties of reserpine.

Methods

Healthy female New Zealand White rabbits weighing 3.0-3.6 kg were used. Before use for fever experiments, these rabbits were trained for a total of 18 h (three 6 h periods) to remain in open ended wooden stocks during which time their body temperature was recorded. Temperatures (±0.05° C) were measured using a Model 44 telethermometer (Yellow Springs Instrument Company) connected to a YSI rectal probe type 401 which was inserted 100 mm into the rectum (Lomax, 1966). At the end of this training period, rabbits whose rectal temperature fell outside of the range 38.8-39.8° C were rejected. All training and fever experiments were carried out in a quiet, well-lit room where the ambient temperature ranged between 20 and 24° C.

For experiments with bacterial pyrogen, the intravenous challenge was provided by 0.1 ml/kg of TAB vaccine (Burroughs, Wellcome & Co), containing 1000 million *S. typhi*, 500 million *S. paratyphi* A and 500 million *S. paratyphi* B in each millilitre. This dose was adhered to throughout this study although it was found that there was little correlation between response and body weight. Indeed, similar results could be obtained by administering 0.3 ml TAB vaccine per rabbit, an observation noted also by Murphy (1967). Stable leucocyte pyrogen fever was achieved according to the method of Cranston, Luff, Rawlins & Rosendorff (1970) using an initial intravenous injection of 10 ml leucocyte pyrogen followed by an infusion at 0.08 ml/minute. Leucocyte pyrogen was obtained from sterile peritoneal exudates prepared according to the method of Bennet & Beeson (1953).

On an experimental day, body temperature was recorded for 60-90 min prior to pyrogen challenge and the mean of the pre-injection temperatures was taken as the base line for the subsequent calculation of fever indices. In the present study the fever responses produced by various treatments were compared by measuring the area underneath a 6 h fever curve. This area was defined as the fever index for that treatment.

Samples of brain tissue for amine assay were dissected from animals which had been killed by an intravenous injection of air. Amines were extracted and assayed as described by Metcalf (1974). Reserpine (Halewood Chemicals Ltd), α-methyltyrosine methyl ester HCl (R. Emanuel Ltd) and *p*-chlorophenylalanine methyl ester HCl (kindly donated by Pfizer, Ltd) were prepared immediately prior to use; doses of the amino acids were expressed as the parent acid in each case.

Results

The effect of pyrogen fever on the concentrations of amines in brain

(a) Bacterial pyrogen Fever was induced in rabbits by the administration of bacterial pyrogen (0.1 ml TAB vaccine/kg i.v.). The biphasic course of the subsequent fever (Figure 1a) was monitored and 3 h after dosage (i.e. at maximum fever) the animals were killed by air embolism and the amine content of the dissected hypothalamus, midbrain and pons/medulla determined (Table 1). The noradrenaline concentration in the hypothalamus was not significantly different from control animals but the concentration of this amine in both the midbrain and pons/medulla was significantly reduced ($P < 0.001$). In contrast, the 5-HT concentration in these latter areas was unaffected although the hypothalamic concentration of 5-HT was significantly reduced ($P < 0.05$). The concentration of dopamine in both the hypothalamus and the midbrain appeared unaffected by bacterial pyrogen fever.

(b) Leucocyte pyrogen A stable, long-lasting fever was induced in rabbits by a 10 ml intravenous injection of leucocyte pyrogen followed by a continuous intravenous infusion at 0.08 ml/minute. Body temperature rose steadily over 1-2 h

and then remained steady at this elevated level (Figure 1b). After 3 h when body temperate had stabilized at the raised level, the animals were killed by air embolism and the requisite brain regions dissected out and analysed for amines (Table 1). Although the concentrations of noradrenaline and 5-HT in the hypothalamus were reduced when compared with controls, in neither case was the reduction statistically significant. In contrast, the concentration of both noradrenaline and 5-HT were significantly reduced in both the midbrain and pons/medulla. Midbrain dopamine was significantly ($P < 0.001$) reduced but the concentration in both the hypothalamus and the pons/medulla was unaffected.

The effect of reserpine on the concentrations of amines and on the magnitude of pyrogen fever

The degree of amine depletion achieved by reserpine (1 mg/kg i.p.) administered to rabbits 17 h before death is shown in Table 1. With the exception of dopamine in the pons/medulla region, all three amines were depleted to a significant extent in all three regions. Rabbits pretreated with reserpine 17 h before challenge with bacterial pyrogen did not exhibit a normal febrile response (Figure 2). Similarly animals pretreated with reserpine 17 h prior to challenge with leucocyte pyrogen also exhibited a significantly diminished pyrogenic response (Figure 3). However animals pretreated with reserpine 42 h prior to leucocyte pyrogen challenge exhibited a normal febrile response to an infusion of leucocyte pyrogen (Figure 3).

The effects of α-methyltyrosine

α-Methyltyrosine (200 mg/kg i.p.) administered as the soluble methyl ester hydrochloride was administered to rabbits 17 h before killing for amine assay. Table 1 shows that whilst 5-HT stores were apparently unaffected by the drug, both noradrenaline and dopamine were significantly depleted in all three areas of brain. Whilst α-methyltyrosine diminished the febrile response to both bacterial (Figure 2) and leucocyte pyrogen (Figure 3) the

Table 1 The effect of various treatments upon the amine concentration in several regions of rabbit brain

Treatment	*Amine*	*Hypothalamus*	*Midbrain*	*Pons/Medulla*
1. None (controls)	Noradrenaline	1.61 ± 0.06	0.73 ± 0.04	0.49 ± 0.03
	Dopamine	0.32 ± 0.03	0.42 ± 0.02	0.15 ± 0.02
	5-HT	1.38 ± 0.18	1.17 ± 0.06	0.50 ± 0.04
2. Bacterial pyrogen (0.1 ml TAB/kg i.v.)	Noradrenaline	1.48 ± 0.15	0.31 ± 0 02†	0.15 ± 0.02†
	Dopamine	0.26 ± 0.03	0.39 ± 0.10	
	5-HT	0.76 ± 0.05**	1.06 ± 0 10	0.47 ± 0.05
3. Leucocyte pyrogen fever (continuous infusion)	Noradrenaline	1.28 ± 0.14	0.27 ± 0.03†	0.27 ± 0.03†
	Dopamine	0.29 ± 0.03	0.23 ± 0.03†	0.13 ± 0.08
	5-HT	1.03 ± 0.11	0.84 ± 0.05**	0.35 ± 0.05**
4. Reserpine (1 mg/kg i.p.)	Noradrenaline	0.32 ± 0.07†	0.07 ± 0.02†	0.09 ± 0.02†
	Dopamine	0.14 ± 0 03**	0.07 ± 0 03†	0.08 ± 0.04
	5-HT	0.17 ± 0.04†	0.21 ± 0.04†	0.09 ± 0.02†
5. α-Methyltyrosine (200 mg/kg i.p.)	Noradrenaline	0.27 ± 0.03†	0.07 ± 0.02†	0.06 ± 0.01†
	Dopamine	0.18 ± 0.03*	0 24 ± 0.03**	0.08 ± 0.01*
	5-HT	1.34 ± 0.05	1.32 ± 0.06	0.59 ± 0.03
6. *p*-Chlorophenylalanine (300 mg/kg i.p.)	Noradrenaline	1.73 ± 0.18	0.56 ± 0.03*	0.37 ± 0.02*
	Dopamine	0.38 ± 0.03	0.48 ± 0.07	0.17 ± 0.03
	5-HT	0.22 ± 0.02†	0.40 ± 0.04†	0.17 ± 0.02†
7. α-Methyltyrosine (200 mg/kg i.p.) + *p*-Chlorophenylalanine (300 mg/kg i.p.)	Noradrenaline	0.24 ± 0.04†	0.08 ± 0.03†	0.08 ± 0 02†
	Dopamine	0.15 ± 0.02**	0.10 ± 0.01†	0.04 ± 0.00**
	5-HT	0.32 ± 0.04**	0.44 ± 0.04†	0.14 ± 0.02

Values are mean values ± s.e. mean expressed as μg/g derived from 6-12 experiments.
* denotes that the value is significantly different from control values at $P < 0.05$.
** denotes significance at $P < 0.01$ and
† denotes significance at $P < 0.001$.

results were only significantly different ($P < 0.05$) from fevers in control animals in the case of leucocyte pyrogen.

The effects of p-*chlorophenylalanine*

Table 1 illustrates that whilst *p*-chlorophenylalanine (300 mg/kg i.p.) administered as the methyl ester hydrochloride 72 h prior to death, significantly lowered the concentration of 5-HT in all three brain regions ($P < 0.001$), it also lowered the concentration of noradrenaline in the brainstem ($P < 0.05$). *p*-Chlorophenylalanine when administered prior to pyrogen challenge did not alter the febrile response to bacterial (Figure 2) or leucocyte pyrogen (Figure 3) to a significant extent, although in the latter case the response was slightly enhanced when compared with control animals.

The combined effect of α-methyltyrosine and p-*chlorophenylalanine*

Seventy-two hours after *p*-chlorophenylalanine (300 mg/kg i.p.) and 17 h after α-methyltryrosine

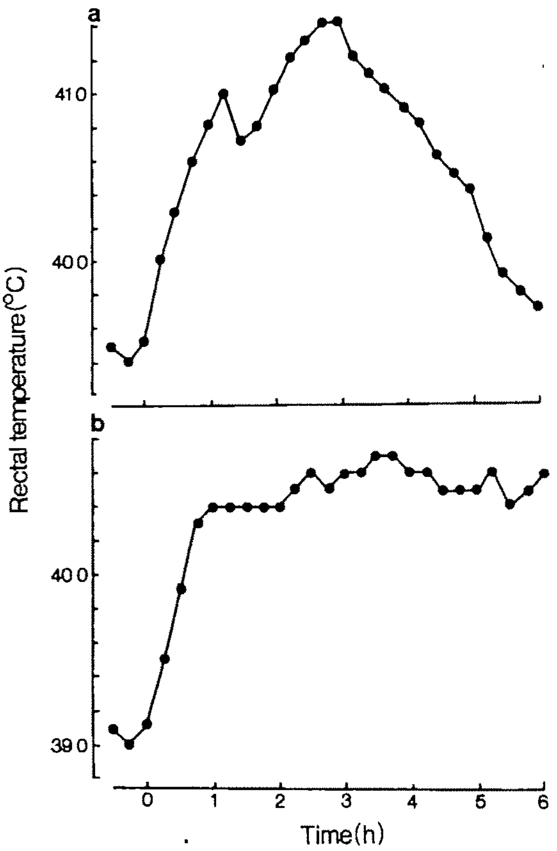

Figure 1 Typical fevers produced by the administration of bacterial or leucocyte pyrogen (a) At zero time, 0.1 ml/kg TAB vaccine injected intravenously, (b) at zero time, 10 ml leucocyte pyrogen injected intravenously followed by a continuous infusion of 0.08 ml/minute.

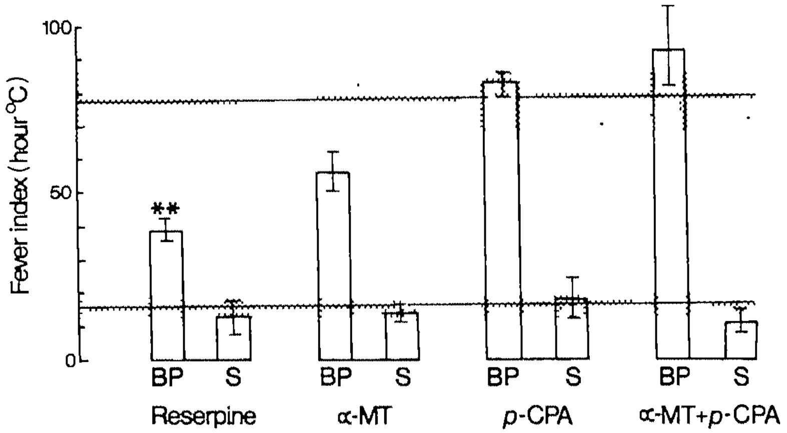

Figure 2 The effect of various drug treatments on the fever induced by injection of bacterial pyrogen (BP) in rabbits. Fever indices are expressed as mean (hours °C) ± s.e. mean obtained from 6-8 animals. The shaded areas represent (mean ± s.e. mean) the fever response of untreated animals to BP challenge (upper) and saline injection (lower). Histograms represent the response to pyrogen challenge (BP) or saline injection (S) after drug pretreatment. Drug dosage and time prior to challenge: reserpine 1 mg/kg i.p., 17 h; α-methyltyrosine (α-MT) 200 mg/kg i.p., 17 h; *p*-chlorophenylalanine (*p*-CPA) 300 mg/kg i.p., 72 h; α-MT + *p*-CPA—combined α-methyltyrosine and *p*-chlorophenylalanine pretreatments. ** Indicates results significantly different from controls at $P < 0.01$.

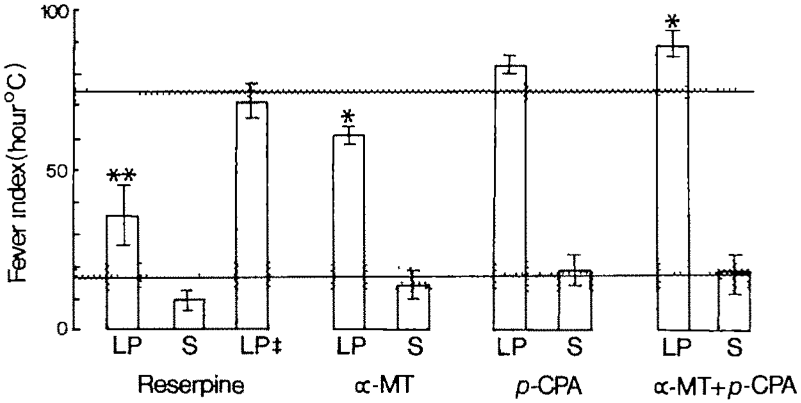

Figure 3 The effect of various drug treatments on the fever induced by a continuous infusion of leucocyte pyrogen (LP). Fever indices are expressed as mean (hours °C) ± s.e. mean obtained from 6-13 animals. The shaded areas represent (mean ± s.e. mean) the fever response of untreated animals to LP challenge (upper) and saline infusion (lower). Histograms represent the response to pyrogen challenge (LP) or saline infusion (S) after drug pretreatment. Drug dosage and time prior to challenge. reserpine 1 mg/kg i.p., 17 h, group marked ‡ received reserpine 42 h before challenge; α-methyltyrosine (α-MT) 200 mg/kg i.p., 17 h; p-chlorophenylalanine (p,-CPA) 300 mg/kg i.p., 72 h; α-MT + p-CPA—combined α-methyltyrosine and p-chlorophenylalanine pretreatments. * and ** indicate results significantly different from controls at $P < 0.05$ and $P < 0.001$ respectively.

Table 2 The effect of amine depleting drugs on rectal temperature in the rabbit prior to pyrogen challenge

Drug	*Dose (mg/kg i.v.)*	*Hours since dosing*	*Rectal temp. °C*
Control	—	—	39.4 ± 0.1
Reserpine	1	17	37.3 ± 0.5*
Reserpine	1	42	38.6 ± 0.1**
α-Methyltyrosine	200	17	38.7 ± 0.1**
p-Chlorophenylalanine	300	72	39.7 ± 0 2
α-Methyltyrosine⁺	200	17	39.2 ± 0.2
p-Chlorophenylalanine	300	72	

Temperatures are mean ± s.e. mean obtained from 6-13 animals * and ** indicate results are significantly different from controls at $P < 0.01$ and $P < 0.001$ respectively.

(200 mg/kg i.p.), both administered as the methyl ester hydrochlorides, a group of rabbits was killed and portions of the brain analysed for amines (Table 1). All three amines were depleted in the three areas analysed. The extent of amine depletion achieved by the combined treatment was not significantly different from that achieved by reserpine. When animals pretreated with both α-methyltyrosine and p-chlorophenylalanine were subsequently subjected to pyrogen challenge the ensuing febrile response was slightly enhanced in both the case of bacterial pyrogen (Figure 2) and leucocyte pyrogen (Figure 3) although only in the latter case was the difference significant ($P < 0.05$).

The effect of amine depleting drugs on body temperature

The effect produced by the various amine depleting treatments prior to pyrogen administration was recorded. The results (Table 2) show that both reserpine and α-methyltyrosine produced significant hypothermia when compared with control animals.

Discussion

Feldberg & Myers (1963) suggested that pyrogen fever is mediated by the release of biogenic amines from the anterior hypothalamus. Since that time observations made by other workers have raised the possibility that the ratio of cations in the hypothalamus (Feldberg, Myers & Veale, 1970) or the release of prostaglandin E_1 (Milton & Wendlandt, 1970) from the same structure may also be important for the chemical mediation of fever.

Several biochemical investigations undertaken since 1963 (Canal & Ornesi, 1961; Kurumma *et al.*, 1964; Takagi & Kuruma, 1966) have supported the original suggestion of Feldberg & Myers by demonstrating that the concentration of 5-HT in the hypothalamus is lowered during bacterial pyrogen fever. The present results confirm that hypothalamic 5-HT is significantly depleted during bacterial pyrogen fever but not during leucocyte pyrogen fever. In contrast, 5-HT in both the midbrain and pons/medulla is reduced during leucocyte but not during bacterial pyrogen fevers. There is therefore no common response of the 5-HT levels to the two pyrogens and since depletion of brain 5-HT by *p*-chlorophenylalanine did not affect the fever produced by the two pyrogens no strong evidence has been obtained for a role of 5-HT in fever heat production.

The effects of the two pyrogens on brain noradrenaline were more comparable. Both pyrogens significantly reduced the concentration of noradrenaline in the brain stem but left that in the hypothalamus unaffected. Also α-methyltyrosine, which depleted noradrenaline in all areas, reduced the fever produced by both pyrogens and itself caused a significant hypothermia. Thus hypothalamic noradrenaline may well be important for the heat production which is achieved in pyrogen fever. A rise in body temperature could then follow the loss of some balancing factor affected by the pyrogens such as midbrain noradrenaline (both pyrogens), hypothalamic 5-HT (bacterial pyrogen) or brain stem 5-HT (leucocyte pyrogen). Such a role for 5-HT is supported by the slight hyperthermia caused by 5-HT depletion after *p*-chlorophenylalanine and it may well be that it is the ratio of 5-HT to noradrenaline in the hypothalamus and brain stem that is important in the control of body temperature as originally suggested by Feldberg & Myers (1963).

Several points arise from the experiments in which the ability of amine depleting drugs to interfere with pyrogen fever was tested. Reserpine suppressed the febrile response to both bacterial and leucocyte pyrogens given 17 h after the drug, but the ability of animals to respond normally to leucocyte pyrogen was restored within 42 hours. However, the role of brain amines in reserpine suppression of fever must be uncertain since after α-methyltyrosine and *p*-chlorophenylalanine together, the febrile responses to both types of pyrogen were enhanced rather than suppressed although the drug combination depleted brainstem amines to the same extent as reserpine. Thus the fever suppression produced by reserpine may depend on pharmacological properties of the molecule other than its ability to deplete tissue stores of catecholamines or 5-HT and the results obtained in these experiments illustrate the dangers of using reserpine as a specific biochemical tool to investigate physiological responses supposedly controlled by biogenic amines. Fever represents a state of abnormal thermoregulation and the present results obtained with amine depleting drugs suggest that the genesis of pyrogen fever is a complex pathophysiological reaction which probably involves other chemical mediators in addition to brainstem monoamines.

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THE EFFECT OF TRICYCLIC ANTIDEPRESSANTS ON CHOLINERGIC RESPONSES OF SINGLE CORTICAL NEURONES

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1 The technique of microelectrophoresis was used in order to study the effects of tricyclic antidepressants on responses of single cortical neurones to acetylcholine.

2 Both potentiation and antagonism of excitatory responses to acetylcholine could be observed after a brief application of imipramine or desipramine. A higher dose of the antidepressant was required to evoke antagonism than to evoke potentiation.

3 Responses to carbachol were affected by desipramine similarly, suggesting that inhibition of cholinesterase is not responsible for the potentiation of cholinergic responses.

4 A brief application of atropine also had a dual effect on responses to acetylcholine.

5 It is suggested that the potentiation of excitatory cholinergic responses by atropine and the antidepressants may be due to the blockade of masked inhibitory receptors.

Introduction

The anticholinergic effects of tricyclic antidepressant drugs in the periphery are well documented (Domenjoz & Theobald, 1959; Atkinson & Ladinsky, 1972), but there is only indirect information concerning a similar action in the brain (Gyermek, 1966). Although an effect on cholinergic systems has generally been regarded as irrelevant so far as the mood elevating effect of these drugs is concerned (Gyermek, 1966), it has been suggested recently that a central anticholinergic effect may also contribute to the therapeutic action of the antidepressants (Janowsky, Davis, El-Yousef & Sekerke, 1972).

We used the technique of microelectrophoresis in order to investigate how responses of single cortical neurones to acetylcholine and to carbachol can be modified by imipramine and desipramine. We also compared the effects of the antidepressants with the effects of atropine. Some of the results presented here have been communicated to the British Pharmacological Society (Bevan, Bradshaw, Roberts & Szabadi, 1973b).

This paper follows another report describing the effects of tricyclic antidepressants on neuronal responses to noradrenaline and 5-hydroxytryptamine (Bradshaw, Roberts & Szabadi, 1974).

Methods

Cats of either sex, weighing 2.0 to 3.5 kg were used. Our methods for the anaesthesia and preparation of animals are described elsewhere (Bradshaw *et al.*, 1974). An area of the anterior or posterior sigmoid gyrus was prepared for recording according to the method of Bradshaw & Szabadi (1972).

Five- or six-barrelled glass micropipettes were constructed and filled as described by Bradshaw, Roberts & Szabadi (1973). Two barrels of each micropipette contained 4 M NaCl, one barrel for recording action potentials, the other for use in current balancing. The remaining barrels contained drug solutions. The drug solutions used in these experiments were acetylcholine chloride (0.2 M, pH 3.6), carbamylcholine (carbachol) chloride (0.2 M, pH 6.9), imipramine hydrochloride (0.2 M, pH 7.4), desmethylimipramine hydrochloride (desipramine) (0.15 M, pH 7.5), and atropine sulphate (0.1 M, pH 5.9).

The techniques used for recording action potentials, and for the electrophoretic application of drugs, were as described by Roberts & Straughan (1967). A cumulative record of the total number of action potentials was obtained via a Grass UI-1 unit integrator.

All the neurones studied were spontaneously active. All the drugs were applied by microelectrophoresis. Repeated responses to acetylcholine (ACh) or carbamylcholine (carbachol) were obtained before and after a brief application of imipramine, desipramine or atropine. Our measure of the dose of the antidepressant was the electrophoretic charge passed (intensity of electro-

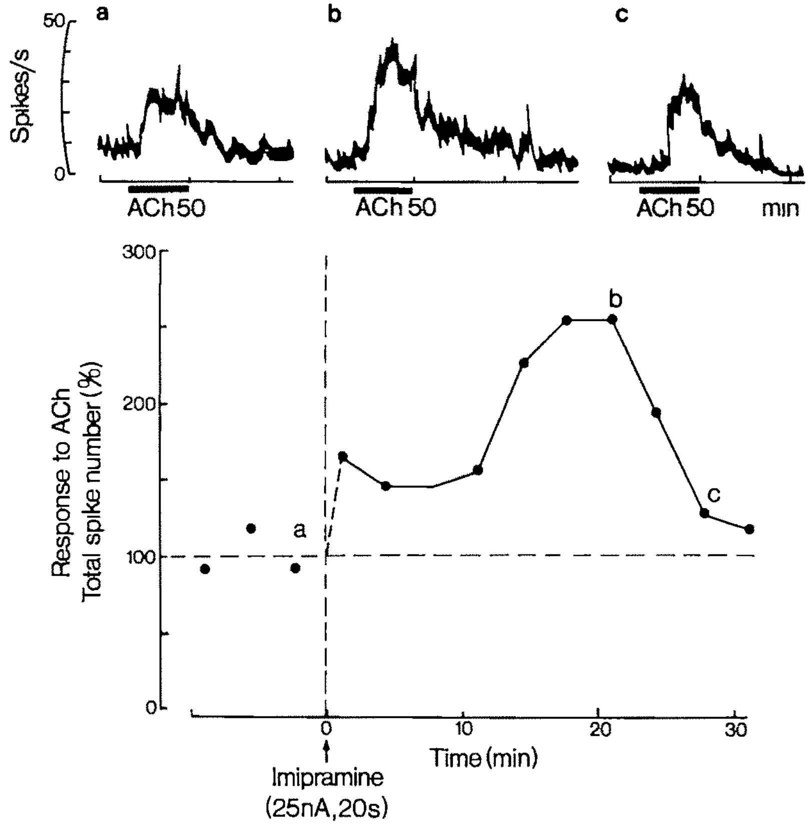

Figure 1 Potentiation of excitatory responses of a single cortical neurone to acetylcholine (ACh) by imipramine. Upper part of figure shows excerpts of the ratemeter recording of the firing rate of the neurone. Horizontal bars indicate applications of ACh; numbers refer to the intensity of the ejecting current (nA). (a) Control response to ACh. (b) Potentiated response to ACh 21 min after a brief application of imipramine (25 nA, 20 seconds). (c) Recovery of control response 28 min after the application of imipramine. Lower graph shows the time-course of the entire study. The sizes of the responses to ACh are expressed as a percentage of the mean of the control responses. Each point represents a single response. Letters above the graph indicate responses illustrated in the ratemeter tracings above.

phoretic current x time of passage of current). The sizes of excitatory responses to the agonists were expressed as the total number of spikes generated in response to each application of an agonist (total spike number) (Bevan, Bradshaw, Roberts & Szabadi, 1973a). The intervals between drug applications were kept constant. During these intervals a retaining current of −25 nA was passed.

Results

Effect of antidepressants on responses to acetylcholine

Imipramine. Drug-interaction studies were successfully completed on 28 neurones responding with a clear increase in firing rate to ACh. Both potentiation and antagonism of the response to ACh could be observed after a brief application (25-100 nA for 20-60 s) of imipramine.

Potentiation of the response was seen in 23 cells. (A response was regarded as potentiated if there was more than a 20% increase over the size of the average control response.) The potentiated response had a characteristic time-course compared to the control response: the peak of the response was higher, and the recovery time longer. The latency of the potentiated response could be either shorter or longer than that of the control response. In a few cells only one response showed potentiation, however, in the majority of cells several responses were potentiated, and the control

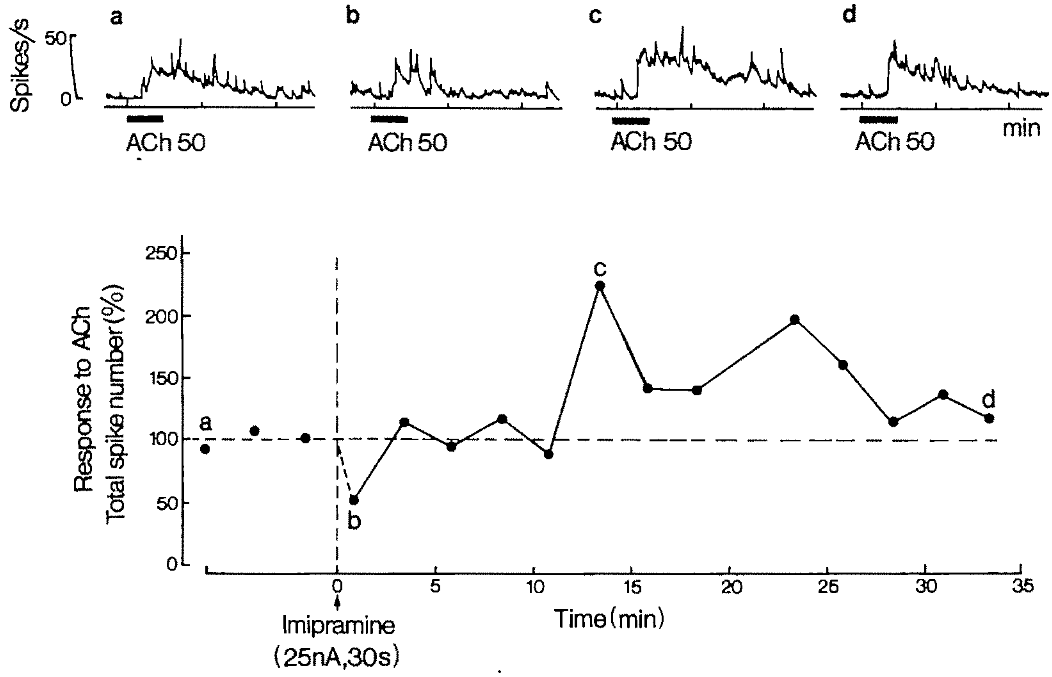

Figure 2 Antagonism and potentiation of excitatory responses of a single cortical neurone to acetylcholine (ACh) by imipramine Upper part of figure shows excerpts of the ratemeter recording of the firing rate of the neurone (as in Figure 1) (a) Control response to ACh. (b) Antagonized response, 1 min after a brief application of imipramine (25 nA, 30 seconds). (c) Potentiated response, 13 min after the application of imipramine. (d) Recovery of control response, 33 min after the application of imipramine. Lower graph shows the time-course of the entire study (as in Figure 1).

response recovered only after a longer time (up to 90 minutes).

Antagonism of the response was seen in 12 cells. Antagonism appeared as a reduction in the total spike number compared to the control. This reduction in size varied between 20-100%.

The occurrence of potentiation and antagonism followed a well-defined time-course. The following patterns could be observed: (1) 'Early' potentiation. In this case the first response after imipramine showed the greatest degree of potentiation. (The first application of ACh after imipramine usually followed less than 1.5 min after the application of imipramine.) Subsequent responses became gradually smaller, until recovery of the control response could be seen. Recovery usually occurred 10-20 min after the antidepressant had been applied. Early potentiation was seen in 10 neurones. (2) 'Late' potentiation. In this case, the potentiation developed gradually, achieving a maximum 10-30 min after the application of imipramine. Recovery occurred 30-60 min after imipramine had been applied. Late potentiation was seen in seven cells. An example of late potentiation is shown in Figure 1. (3) Antagonism followed by potentiation. In this case, the first response after imipramine was reduced in size. This initial antagonism was later followed by potentiation, and finally by gradual recovery of the control response. This pattern of drug-interaction was observed in six cells. An example is shown in Figure 2. (4) Antagonism only was seen in six cells. In this case, an initial antagonism of the response was followed by recovery. In some cells a number of studies were conducted using more than one dose of imipramine, and more than one pattern of drug interaction could be observed.

When both antagonism and potentiation were observed (pattern 3), antagonism invariably preceded potentiation (e.g. Figure 2), and the reverse was never seen. This suggested that antagonism appeared when the concentration of imipramine at the cell was likely to have been at its highest, that is immediately following the electrophoretic ejection of the antidepressant. In order to test this hypothesis, we investigated the correlation between the dose of imipramine applied, and the occurrence of antagonism or potentiation. The

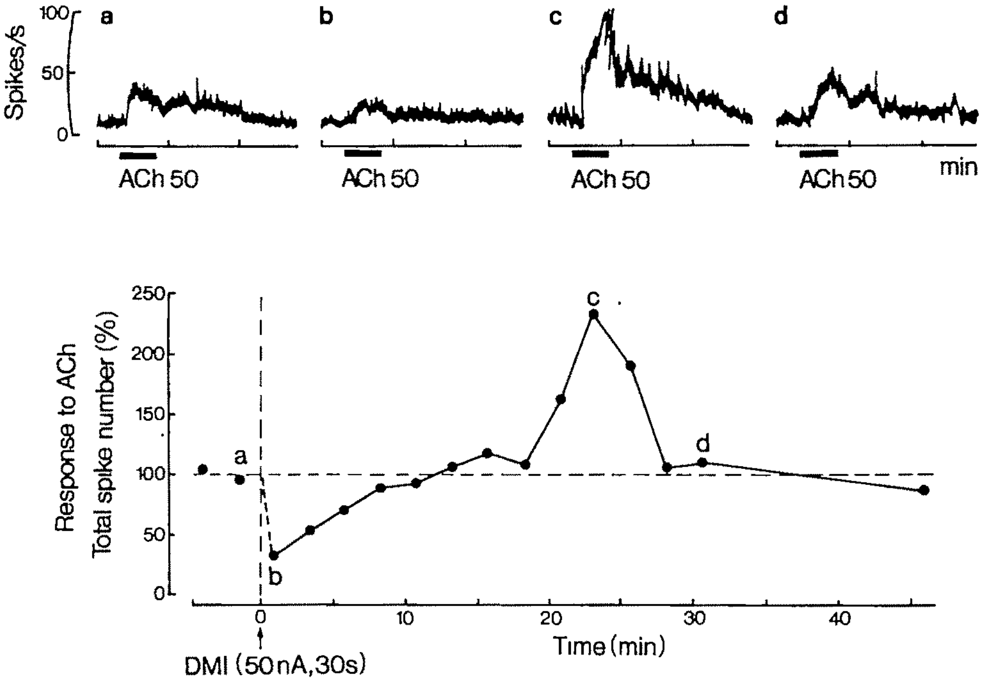

Figure 3 Antagonism and potentiation of excitatory responses of a single cortical neurone to acetylcholine (ACh) by desipramine (DMI). Upper part of figure shows excerpts of the ratemeter recording of the firing rate of the neurone (as in previous figures). (a) Control response to ACh. (b) Antagonized response, 1 min after a brief application of desipramine (50 nA, 30 seconds). (c) Potentiated response, 23 min after the application of desipramine. (d) Recovery of control response, 30 min after the application of desipramine. Lower graph shows the time-course of the entire study (as in previous figures).

results are summarized in Table 1. It is apparent that a lower dose of imipramine (0.4-2.0 μC) was more likely to cause potentiation only, whereas a higher dose ($\geqslant$2.0 μC) was more likely to result in antagonism as well (χ^2 test: $P < 0.01$).

Desipramine. The effect of desipramine was studied in 21 cells. Both potentiation and antagonism of the responses could be observed after a brief application of desipramine. The same patterns of drug-interaction were seen as with imipramine. (1) 'early' potentiation (four cells); (2) 'late' potentiation (four cells); (3) antagonism followed by potentiation (10 cells); (4) antagonism only (seven cells). An example of antagonism followed by potentiation is shown in Figure 3.

Effect of desipramine on responses to carbachol

In an attempt to test the hypothesis that potentia-

Table 1 Relationship between dose of imipramine applied and effect on responses to acetylcholine

	Number of cells	
Dose (charge)	*Potentiation only*	*Antagonism**
0.4-2.0 μC	17	4
>2.0 μC	2	5

$\chi^2 = 9.2034; P < 0.01$

* Includes all cells in which antagonism was seen. In some of these cells the antagonism was later followed by potentiation.

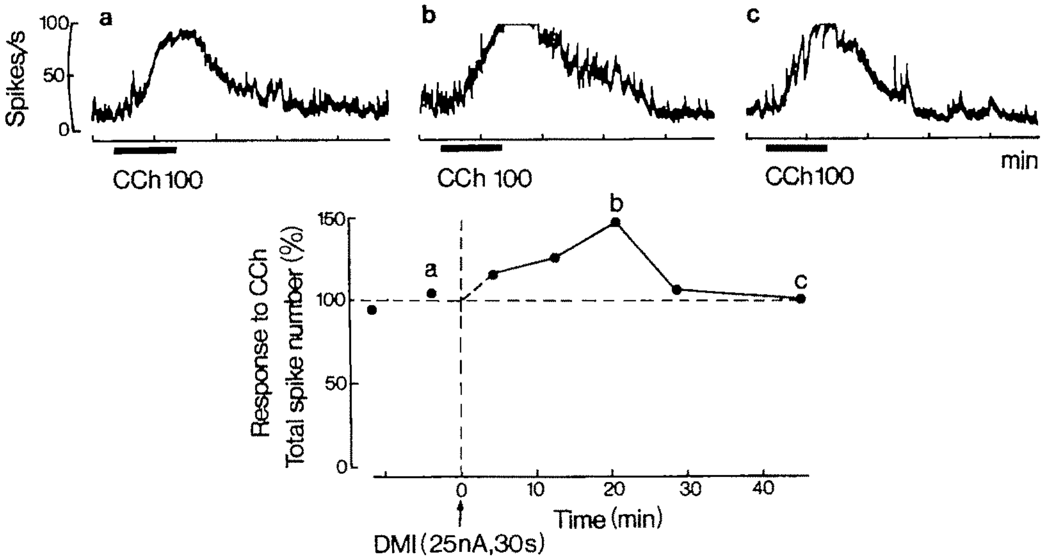

Figure 4 Potentiation of excitatory responses of a single cortical neurone to carbachol (CCh) by desipramine (DMI). Upper part of figure shows excerpts of the ratemeter recording of the firing rate of the neurone (as in previous figures). (a) Control response to carbachol. (b) Potentiated response 20 min after a brief application of desipramine (25 nA; 30 seconds). (c) Recovery of control response, 44 min after the application of desipramine. Lower graph shows the time-course of the entire study (as in previous figures).

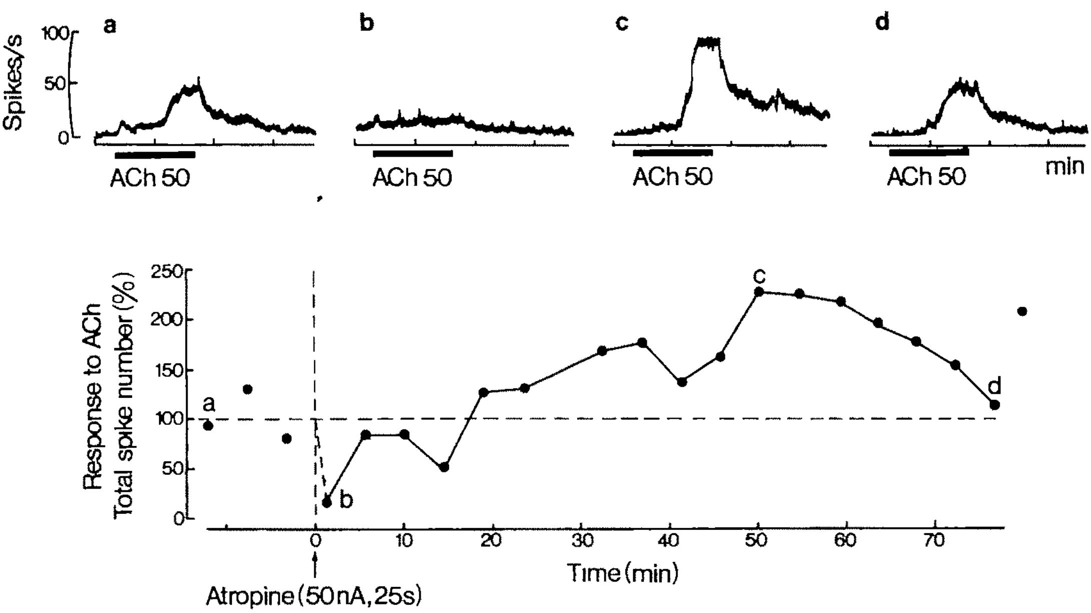

Figure 5 Antagonism and potentiation of excitatory responses of a single cortical neurone to acetylcholine (ACh) by atropine. Upper part of figure shows excerpts of the ratemeter recording of the firing rate of the neurone (as in previous figures). (a) Control response to ACh. (b) Antagonized response, 1 min after a brief application of atropine (50 nA; 25 seconds). (c) Potentiated response, 50 min after the application of atropine. (d) Recovery of control response, 76 min after the application of atropine. Lower graph shows the time-course of the entire study (as in previous figures).

tion of responses to ACh is due to the inhibition of the activity of cholinesterase (Osborne & Sigg, 1960), we have examined how responses to carbachol are influenced by desipramine. It is known that carbachol is not hydrolysed by cholinesterase (Goodman & Gilman, 1970).

The effect of desipramine on excitatory responses to carbachol was studied in 10 neurones. In six cells responses to carbachol were potentiated by desipramine; in three of these cells the potentiation was preceded by antagonism. In the remaining four cells, antagonism alone was seen. An example of potentiation is shown in Figure 4.

Effect of atropine on responses to acetylcholine

As the results with imipramine and desipramine suggested that a smaller dose of the antidepressant was needed to potentiate than to antagonize the response to ACh, we have examined the effect of a similar small dose of atropine on the responses to ACh in 14 cells. Potentiation was observed in 11 cells: in one cell potentiation alone was seen, whereas in 10 cells the potentiation was preceded by antagonism. In three cells antagonism only could be observed. An example of the dual effect of atropine is shown in Figure 5.

Discussion

The results show that imipramine and desipramine have a dual effect on responses of single cortical neurones to acetylcholine: both antagonism and potentiation can be observed. Moreover, this effect is dose-dependent: smaller doses potentiate and bigger doses antagonize the responses. This dual effect may reflect the operation of two independent mechanisms: a more sensitive 'potentiating' mechanism, and a less sensitive 'antagonizing' mechanism. According to this model, a lower concentration of the antidepressant at the cellular receptor sites would affect the more sensitive potentiating mechanism only, whereas a higher concentration would activate the antagonizing mechanism as well. The size of any particular response to ACh would be determined by the interaction between the two mechanisms, whereas the time-course of the drug-interaction patterns (e.g. antagonism followed by potentiation) would reflect a gradual decline in the concentration of the antidepressant at the neurone after the application of the antidepressant had been terminated (Bradshaw *et al.*, 1974).

The most plausible explanation for the antagonism is the blockade of muscarinic receptors by the antidepressant. There is a considerable amount of experimental information suggesting that the excitatory responses of cortical neurones to ACh may reflect the activation of muscarinic receptors (see Curtis & Crawford, 1969) Furthermore, it is well documented that tricyclic antidepressants can block muscarinic receptors in the periphery (Domenjoz & Theobald, 1959, Atkinson & Ladinsky, 1972).

It is more difficult to interpret the potentiation of responses to acetylcholine. Apart from one early report (Osborne & Sigg, 1960), this effect has not been studied in the periphery. Osborne & Sigg (1960) reported a dual effect a smaller dose of imipramine potentiated, whereas a bigger dose antagonized some peripheral effects of acetylcholine. These authors suggested that the potentiating effect was due to the blockade of cholinesterase, since they could demonstrate an inhibitory effect of imipramine on serum cholinesterase. However, this is not a likely explanation for the potentiation seen in our experiments, since the activity of brain acetylcholinesterase is virtually unaffected by tricyclic antidepressants (Perkinson, Ruckart & DaVanzo, 1969). Moreover, our observation that responses to carbachol are also potentiated by desipramine confirms that potentiation is not due to the inhibition of cholinesterase activity, since cholinesterase does not play a role in the elimination of carbachol (Goodman & Gilman, 1970).

As cholinesterase blockade cannot explain potentiation in our system, we would like to propose an alternative explanation. It has been shown that both excitatory and inhibitory receptors to ACh can co-exist on neurones in molluscs (Kehoe, 1972). Furthermore, it has been suggested that a similar situation may occur on some mammalian neurones (Barker, Crayton & Nicoll, 1971). The occurrence of both excitatory and depressant responses of cortical neurones to ACh (see Curtis & Crawford, 1969) would suggest that both excitatory and inhibitory receptors to ACh may occur on these cells. Since in our experiments cortical neurones were invariably excited by ACh, the inhibitory receptors would seem to have been masked, their activation causing only a reduction in the size of the excitatory response. We propose that potentiation may be due to the selective blockade of masked inhibitory receptors by a smaller concentration of the antidepressant. A higher concentration of the antidepressant would block the dominant excitatory receptors as well, thus causing a reduction in the size of the observed response. Our observation of the dual action of atropine would be consistent with this hypothesis: a smaller concentration of atropine would block the masked inhibitory receptors selectively, thus causing potentiation of the response. This potentiating effect of a low

Table 2 Effect of antidepressants and antagonists on responses of single cortical neurones to potential neurotransmitters

	Noradrenaline	*5-Hydroxytryptamine*	*Acetylcholine*	*Glutamate*
Imipramine	P, A (1)	P, A (1)	P, A	O (1)
Desimpramine	P, A (1)	P, A (1)	P, A	O (1)
Sotalol	P, A (2)	P, A (2)	O (2)	O (4)
Methysergide	P, A (2)	P, A (2)	O (2)	O (3)
Atropine	O (4)		P, A	O (5)

P = potentiation, A = antagonism, O = not affected.
(1) Bradshaw, Roberts & Szabadi (1974). (2) Bevan, Bradshaw & Szabadi (1974) (3) Roberts & Straughan (1967). (4) Johnson, Roberts, Sobieszek & Straughan (1969) (5) Stone (1972).

dose of atropine has not been described before in single cell pharmacology in the central nervous system. It has been described in peripheral preparations, however, where a low concentration of atropine may cause 'paradoxical' potentiation of responses to ACh (Arunlakshana & Schild, 1959).

An alternative explanation for the potentiating effect of atropine on single neurones could be that it acts by blocking the uptake of ACh into pre-synaptic terminals. It has been reported that ACh is taken up actively into brain slices in the presence of irreversible cholinesterase inhibitors, and that this uptake is inhibited by atropine (Liang & Quastel, 1969). There is no evidence, however, that ACh uptake occurs *in vivo* when cholinesterase has not been inhibited (Katz & Chase, 1971).

A similar dual action of the tricyclic antidepressants has been described on neuronal responses to noradrenaline and 5-hydroxytryptamine (Bradshaw *et al.*, 1974). Moreover, it has been reported that some peripheral antagonists of noradrenaline and 5-hydroxytryptamine also have a dual action on responses to monoamines (Bevan, Bradshaw & Szabadi, 1974). The hypothesis of the two opposite receptors has been used to account for these observations (Szabadi & Bradshaw, 1974). The effects of antagonists and antidepressants on cortical neuronal responses to potential neurotransmitters are summarized in Table 2. It is apparent that while atropine seems to be a specific blocker of ACh receptors, and sotalol and methysergide are specific in blocking monoamine receptors, the tricyclic antidepressant can act at both acetylcholine and monoamine receptors.

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SOME CENTRAL EFFECTS OF 5-HYDROXYTRYPTAMINE IN YOUNG CHICKENS AT AND BELOW THERMONEUTRALITY

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1 Three salts of 5-hydroxytryptamine, the hydrogen maleinate, the oxalate and the creatinine sulphate were infused into the hypothalamus of 10-18 day old chickens at ambient temperatures in and below the thermoneutral range Body temperature was recorded and behaviour observed. Electrocortigrams were recorded in experiments in which 5-hydroxytryptamine hydrogen maleinate was used. The effects of a monoamine oxidase inhibitor and methysergide on these responses were similarly studied.

2 At thermoneutrality (31° C) all 3 salts produced behavioural sleep. 5-Hydroxytryptamine oxalate had inconsistent effects on body temperature. 5-Hydroxytryptamine creatinine sulphate produced hypothermia at small doses and mild hyperthermia at higher doses. 5-Hydroxytryptamine hydrogen maleinate produced hypothermia at all doses tested; the falls in temperature induced by this salt were intensified in magnitude and duration by monoamine oxidase inhibition unlike the responses to the other 2 salts.

3 At temperatures below thermoneutrality (16° C) all 3 salts produced behavioural sleep and electrocortical sleep was recorded with 5-hydroxytryptamine hydrogen maleinate. All 3 salts produced hypothermia, which was intensified in magnitude and duration by monoamine oxidase inhibition.

4 The hypothermia produced by 5-hydroxytryptamine hydrogen maleinate was prevented by equimolar doses of methysergide.

5 The position of the cannula in the hypothalamus was found to be crucial.

6 The results contrast with those found in the adult fowl. No conclusion is drawn as to the relationships of the actions of these salts when infused compared with the effects of endogenous 5-hydroxytryptamine release.

Introduction

Whereas 5-hydroxytryptamine hydrogen maleinate infused into the hypothalamus of adult fowls elevated body temperature (Marley & Nisticò, 1975), in young chickens the reverse effect was obtained, viz, 5-hydroxytryptamine hydrogen maleinate lowered body temperature. Bligh & Cottle (1969) observed for species in which 5-hydroxytryptamine increased heat loss and decreased heat production at thermoneutrality, that these effects were intensified at lower ambient temperatures. This applied also for young chicks but was unlikely to be true for adult fowls. Sleep was also induced by infusions of 5-hydroxytryptamine irrespective of whether the chicks were tested at or below thermoneutrality, and was more intense than in adult fowls despite smaller dosage. 5-Hydroxytryptamine hydrogen maleinate was taken as the criterion agonist, and its effects compared with those of the oxalate and creatinine sulphate species.

Methods

The techniques used for stereotactic implantation, under halothane anaesthesia, of an infusion cannula into the hypothalamus of 11-16 day chicks (body weight approximately 80 g) and for implantation of a thermistor and cortical recording electrodes have been described by Marley & Stephenson (1970). Chickens were not investigated until at least 24 h after recovery from the operative procedures, and only those which had fed since the operation, as indicated by the presence of food in the crop, were used. The chicks were tested in a sound-proofed controlled-environment chamber (Stephenson, 1971) ambient temperature in the chamber being maintained respectively below (16 ± 0.5°C) or within (30°-31°C) the thermoneutral range for chicks of this age; relative humidity was approximately 60 per cent. The methods used for remote infusion of drugs into the hypothalamus (1.0 μl in 2 min) or intravenously, and for recording and integrating electrocortical activity have also been described (Marley & Stephenson, 1970). After the experiments, the brains were preserved in formol saline for subsequent staining and histological location of the positions of the cannulae.

Drugs

These included the hydrogen maleinate, oxalate and creatinine sulphate salts of 5-hydroxytryptamine; mebanazine oxalate, and methysergide bimaleate.

Results

In all experiments, 5-hydroxytryptamine was infused into the hypothalamus.

5-Hydroxytryptamine hydrogen maleinate

Thermoneutral ambient temperature 5-Hydroxytryptamine, 0.05 μmol (6 chicks) induced sleep and lowered body temperature 0.8° to 1.3°C (Figure 1a), the fall in body temperature starting immediately after infusion and reaching a nadir after 10-15 min, with recovery in about 60 min; body temperature was then elevated 0.2° to 0.3°C above control values for at least 60 min (Figure 1a). Two chicks were then given 5-hydroxytryptamine, 0.2 μmol, which lowered body temperature 1°C, with recovery after 110 minutes. Four further chicks were pretreated with mebanazine, 10 μmol/100 g intravenously 24 h and 1 h before giving 5-hydroxytryptamine. (These intravenous doses, and their timing in relation to 5-hydroxytryptamine infusions were employed in subsequent experiments with mebanazine). Body temperature fell 0.5° to 1.0°C after the second dose of mebanazine (Figure 2a). Once body temperature had stabilized 5-hydroxytryptamine, 0.5 μmol, lowered body temperature a further 1.5°C (Figure 2a); the nadir was reached later (at 40 min) than without mebanazine, and there was delayed recovery (compare Figure 2a with 1a).

Ambient temperature below thermoneutrality 5-Hydroxytryptamine 0.05 μmol, lowered body temperature (9 chicks) considerably more than at thermoneutrality. Body temperature had fallen 8.0°C, 20 to 30 min after 5-hydroxytryptamine, with recovery in 160 min and subsequent elevation, for at least 60 min, of body temperature to 1.0°C above control values (Figure 1b). Sleep was intense and extended beyond recovery of body temperature. The change from the control alert electrocortical pattern (Figure 1d) to that during sleep, 15 min after 5-hydroxytryptamine, 0.025 μmol, is shown in Figure 1e together with

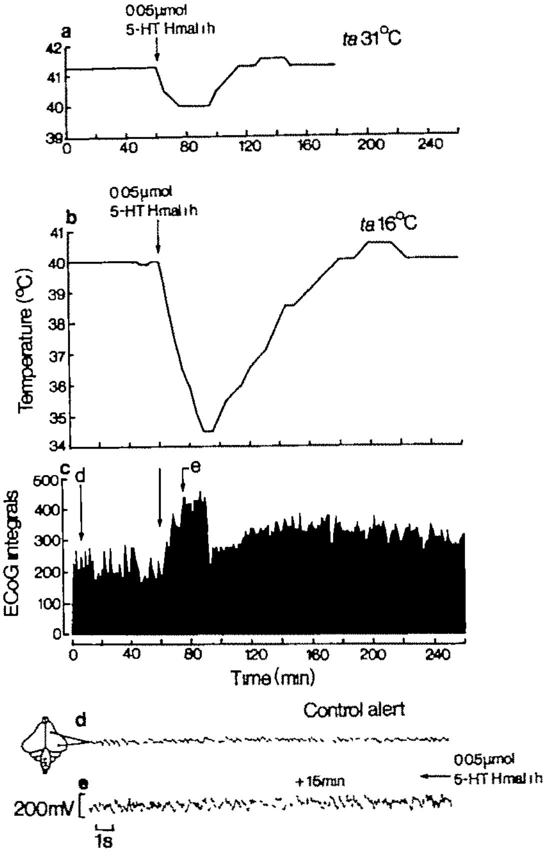

Figure 1 Effects of 5-hydroxytryptamine hydrogen maleinate (5-HT Hmal), 0.05 μmol, infused into the hypothalamus (i.h.) of chicks aged 11 (a) and 16 days (b, c, d, e). (a) Lowering of the body temperature after 5-hydroxytryptamine, the chick at an ambient temperature (*ta*) of 31°C. (b) Much greater hypothermic effect of 5-hydroxytryptamine at an ambient temperature of 16°C. (c) Histogram of integrated electrocortical activity obtained throughout the experiment in (b); epochs of electrocortical activity were sampled at (d) and (e). (d) Illustrates the alert control electrocortical activity, and (e) electrocortical sleep 15 min after infusion. Positions of the recording electrodes on the brain are also shown.

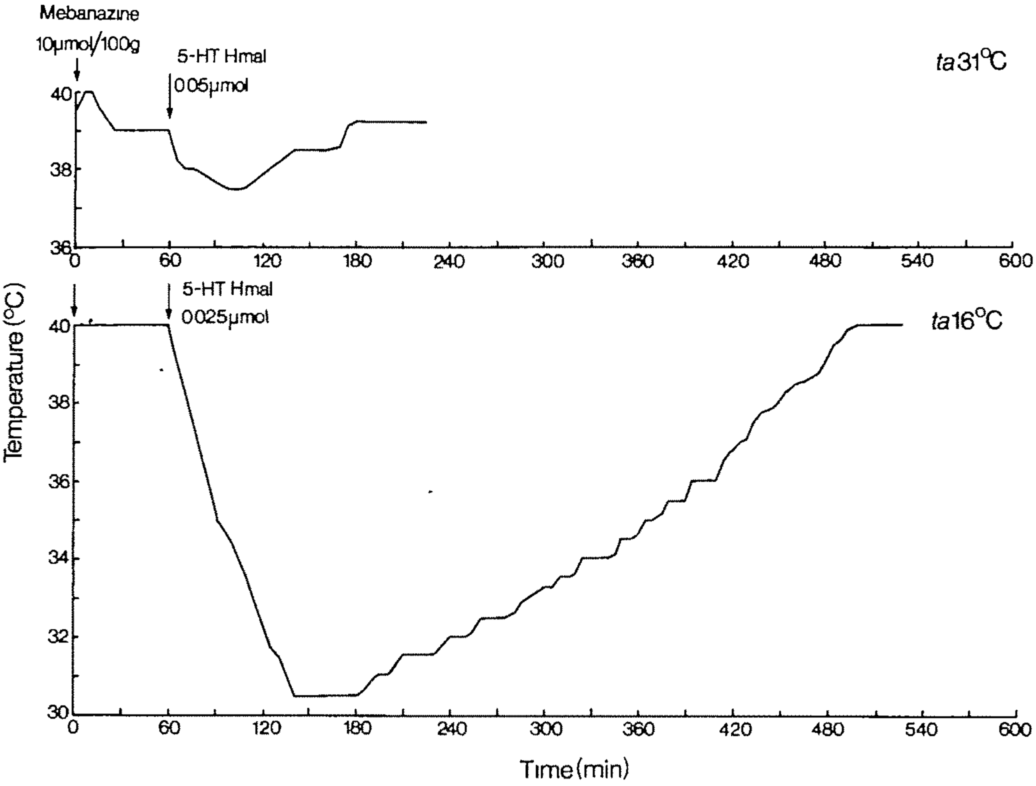

Figure 2 Effect of infusing 5-hydroxytryptamine hydrogen maleinate (5-HT Hmal) into the hypothalamus of a 16 day (84 g) chick, after two intraperitoneal injections of mebanazine oxalate, 10 µmol/100 g, one given 24 h before and one 1 h before infusion, on body temperature (a) Shows the effect of 0.05 µmol at an ambient temperature (*ta*) of 31° C; (b) shows the effect of 0.025 µmol at an ambient temperature of 16° C.

the accompanying increases in electrocortical integrals (Figure 1c), an increase sustained after recovery of body temperature. Methysergide (0.05 µmol/100 g, i.v., 10 min previously), prevented both the sleep and fall in body temperature to this dose of 5-hydroxytryptamine (3 chicks). Pretreatment with mebanazine (7 chicks) prolonged and enhanced the duration and fall of body temperature evoked by 5-hydroxytryptamine, 0.025 µmol (compare Figures 2b and 1b); sleep was also intensified. In 3 chicks, 0.05 µmol, 5-hydroxytryptamine was infused after mebanazine pretreatment. This produced lethal falls in body temperature.

5-Hydroxytryptamine oxalate

Thermoneutral ambient temperature 5-Hydroxytryptamine 0.02 µmol (2 chicks) or 0.05 µmol (2 chicks) did not significantly alter body temperature; nevertheless, the chicks slept following 5-hydroxytryptamine but were readily aroused. Similar results were obtained in 4 chicks pretreated with mebanazine.

Ambient temperature below thermoneutrality
5-Hydroxytryptamine, 0.02 µmol (2 chicks) lowered body temperature 1.0° C with recovery in about 30 min, and subsequently elevated body temperature by 0.5° C for 40 min above control values; the chicks were asleep throughout this period. The hypothermic effect of a larger dose of 5-hydroxytryptamine, 0.05 µmol (2 chicks) was very marked, body temperature declining up to 11.75° C with recovery incomplete even 510 min later; deep sleep was induced by 5-hydroxytryptamine. The effects of 5-hydroxytryptamine were potentiated by pretreatment with mebanazine. After such pretreatment, 5-hydroxytryptamine, 0.02 µmol (2 chicks) lowered body temperature 7.0° C (compared with 1.0° C without mebanazine), the nadir occurring after 60 min and

recovery at 255 min, with subsequent elevation of body temperature, 1.25° C, above pre-infusion values

5-Hydroxytryptamine creatinine sulphate

Thermoneutral ambient temperature 5-Hydroxytryptamine (2 chicks) 0.02 μmol, infused into the hypothalamus induced profound sleep but produced inconsistent changes in body temperature. Infusion of 5-hydroxytryptamine, 0.05 μmol, into four chicks produced sleep and body temperature fell 2 5° to 4° C, for 45-60 min before recovery to control values. In two chicks in which 5-hydroxytryptamine, 0.05 μmol, produced sleep but no change in body temperature, the cannula was found to be more anterior than usual. 5-Hydroxytryptamine, 0.2 μmol (2 chicks) produced a 0.5° C rise in body temperature lasting 90 min, with a delay of 20 min before onset.

Ambient temperature below thermoneutrality 5-Hydroxytryptamine, 0.01 or 0.05 μmol lowered body temperature 2.0° to 3.5° C in 3 chicks but was ineffective in a fourth. Maximum fall of body temperature occurred 30 min after infusion, with recovery 50 min later. After pretreatment with mebanazine (1 chick), 5-hydroxytryptamine, 0.05 μmol now lowered body temperature 10.0° C. In all these experiments, 5-hydroxytryptamine induced intense sleep.

Discussion

Effects of the maleinate, oxalate and creatinine sulphate salts of 5-hydroxytryptamine infused into the chick hypothalamus, on body temperature, were determined. At thermoneutrality, 5-hydroxytryptamine hydrogen maleinate lowered body temperature whereas the oxalate lacked effect. Responses to 5-hydroxytryptamine creatinine sulphate were inconsistent, small doses, 0.05 μmol, lowering body temperature, whereas larger doses, 0.2 μmol, elevated it. A reduction in ambient temperature potentiated hypothermia to 5-hydroxytryptamine hydrogen maleinate and creatinine sulphate, 5-hydroxytryptamine oxalate producing a biphasic response (initial fall then rise). Behavioural sleep was induced by all three salts. Since the work of Roberts & Straughan (1967) and Bradley (1968) on cat cortical and brain-stem neurones indicated that the maleinate ion applied iontophoretically did not influence neuronal firing-rate, 5-hydroxytryptamine hydrogen maleinate was taken as the paradigm.

The effects of 5-hydroxytryptamine hydrogen maleinate infused into the hypothalamus differed in young chicks from those obtained in adult fowls. First, effective doses of 5-hydroxytryptamine hydrogen maleinate were one quarter of those needed in the adult (0.2 μmol) a difference possibly accounted for by the larger size of the hypothalamus in adult fowls (brain volumes of adult and young fowls approximately 5.0 ml and 1.5 ml, respectively). Second, 5-hydroxytryptamine hydrogen maleinate elevated body temperature 1-3° C in adult fowls at thermoneutrality (31° C) but lowered it, 1.5° C in young chicks. This difference could not be ascribed to the differences in doses in adult compared with young chickens, since the adult dose (0.2 μmol) also produced a fall in body temperature of 1° C in young chicks.

Although hypothermic effects of the 5-hydroxytryptamine salts were more pronounced below thermoneutrality, an ambient temperature of 16° C is not a severe cold stress since chicks have been maintained for 24 h at this temperature and in a relative humidity of 50-60% without alteration of normal body temperature or electromyographic evidence of shivering (Marley & Stephenson, 1975). Also, continuous electrocortical and behavioural observation over 48 h revealed no change in the sleep-waking continuum at 16° C compared with thermoneutrality (Whelan, unpublished data).

Compatible with the pharmacological properties of 5-hydroxytryptamine, effects were potentiated by prior amine oxidase inhibition; the monoamine oxidase inhibitor itself lacked significant effects on behaviour, and so did not interfere with the interpretation of the results. Although tested solely against 5-hydroxytryptamine hydrogen maleinate, the hypothermic effects were prevented by methysergide, a 5-hydroxytryptamine and tryptamine antagonist.

Insufficient data were available to clarify whether the effects of 5-hydroxytryptamine salts on sleep and body temperature could be ascribed to actions at different nuclei in the chick; although phenomenological separation was possible in the adult (Marley & Nisticò, 1975). However, in the 2 chicks in which sleep was elicited but little or no temperature effects were evoked by 5-hydroxytryptamine creatinine sulphate, the cannula position was found to be in the preoptic area of the anterior hypothalamus. Also the maximum temperature effect was elicited with 5-hydroxytryptamine hydrogen maleinate when the cannula tip was in the posterior hypothalamus.

Since the effects described result from the sum of drug action on neurone populations and subsequent local changes in ionic environment, parameters difficult to quantify, a complete explanation cannot be offered for the differences in responses to the 5-hydroxytryptamine salts nor is it possible to decide which of the drugs produced effects most representative of the endogenous transmitter.

This work was undertaken in partial fulfilment of the requirements for the degree of PhD in the University of London by J.E.W. who is a M.R.C. scholar. We are grateful to Sandoz for the methysergide and to G. Garcia for doing the histology.

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POSSIBLE ROLE OF A β-ADRENOCEPTOR IN THE REGULATION OF NORADRENALINE RELEASE BY NERVE STIMULATION THROUGH A POSITIVE FEED-BACK MECHANISM

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1 The effects of isoprenaline, propranolol and phentolamine, were studied on tritiated noradrenaline overflow elicited by postganglionic nerve stimulation in guinea-pig isolated atria.

2 Isoprenaline (1.2×10^{-8}M) increased while propranolol (1.0×10^{-7}M) reduced the overflow of tritiated noradrenaline evoked by nerve stimulation. These effects were less than those of phentolamine (3.1×10^{-6}M), which increased by approximately three-fold the overflow of [^{3}H]-noradrenaline elicited by nerve stimulation.

3 Neuronal accumulation of tritiated noradrenaline in guinea-pig atria was not affected by isoprenaline, propranolol or phentolamine at the concentrations employed in this study.

4 Isoprenaline (1.2×10^{-8}M) induced a positive chronotropic effect of about 80% of the maximum. On the other hand, propranolol produced a shift to the right in the frequency-response curve to nerve stimulation and in the concentration-response curve to exogenous noradrenaline in guinea-pig atria.

5 In the isolated nictitating membrane of the cat, the frequency-response curve to nerve stimulation was not modified by propranolol, while in the presence of 3.9×10^{-6}M of *N*,-2-(2,6-dimethylphenoxy)propyl-*N*,*N*,*N*-trimethylammonium (β-methyl-TM 10) there was a shift to the right and a depression of slope. Neither propranolol nor β-methyl-TM 10 affected responses to exogenous noradrenaline.

6 The effects of isoprenaline and of propranolol on transmitter release are compatible with the view that in addition to the presynaptic negative feed-back mechanism for noradrenaline release by nerve stimulation mediated via α-adrenoceptors a positive feed-back mechanism exists in adrenergic nerve endings which is triggered through the activation of presynaptic β-adrenoceptors.

Introduction

The release of noradrenaline by nerve stimulation appears to be regulated through a negative feed-back mechanism mediated by presynaptic α-adrenoceptors (Langer, Adler, Enero & Stefano, 1971, Farnebo & Hamberger, 1971; Kirpekar & Puig, 1971; Enero, Langer, Rothlin & Stefano, 1972; Starke, 1972a). According to this hypothesis, once the transmitter released by stimulation reaches a threshold concentration in the synaptic gap, it activates presynaptic α-adrenoceptors, triggering a negative feed-back mechanism that inhibits further release of the transmitter. The experimental evidence that led to postulation of this mechanism is based on the inhibition of transmitter release in the presence of α-adrenoceptor agonists (Starke, 1972a; Starke, 1972b) and the increase in transmitter release obtained in the presence of α-adrenoceptor blocking agents (Langer, 1970, Farnebo & Hamberger, 1971; Starke, Montel & Schümann, 1971; Enero *et al.*, 1972).

On the other hand, there is little information about the effects of β-adrenoceptor agonists and antagonists on transmitter released by nerve stimulation, although propranolol and other β-adrenoceptor blocking agents are used in the treatment of hypertension (Dorph & Binder, 1969; Prichard & Gillam, 1969; Tibblin & Åblad, 1969; Zacharias & Cowan, 1970; Thorpe, 1972) and it appears that inhibition of release of adrenergic transmitter may contribute to their hypotensive effect (Mylecharane & Raper, 1970; Eliash & Weinstock, 1971).

Consequently, it was of interest to study the effects of isoprenaline and propranolol on [^{3}H]-noradrenaline release evoked by accelerans nerve stimulation in guinea-pig isolated atria.

Methods

Guinea-pig atria

Guinea-pigs of 300 to 500 g of either sex were anaesthetized with sodium pentobarbitone (35 mg/kg i.p.). The hearts were removed and both atria were dissected together with the accelerans nerve, in Locke solution of the following composition (mM): NaCl, 154.0; KCl, 5.6; $CaCl_2$, 2.2; $NaHCO_3$, 6.0; glucose, 11.1; ethylenediamine tetraacetic acid disodium salt (EDTA), 0.04 and ascorbic acid, 0.11. Atropine, 1.4×10^{-6}M was added to the Locke solution which was bubbled continuously with O_2. The atria, together with the accelerans nerve were set up in an isolated organ bath fitted with platinum electrodes for nerve stimulation. The tissue was attached to a force displacement transducer connected to a Grass polygraph to record spontaneous contractions of the preparation. All experiments were carried out in oxygenated Locke solution at 37°C.

In experiments in which the noradrenaline stores were labelled, the atria were incubated for 30 min with 10 µCi/ml of (±)-[7-^{3}H]-noradrenaline (New England Nuclear Corporation, Boston, Mass) as described previously by Adler-Graschinsky, Langer & Rubio (1972).

The accelerans nerves were stimulated at 4 Hz for 60 s with square pulses of 0.5 ms duration and supramaximal voltage. In these experiments, two periods of nerve stimulation were applied. The first period of stimulation (S_1) was applied 85 min after the end of the incubation of the atria with tritiated noradrenaline. The second stimulation (S_2) was applied 40 min later.

The total increase in outflow of tritium elicited by nerve stimulation was expressed as the fractional release per shock (FR), i.e. the total nCi released per shock divided by the total nCi remaining in the tissue at the onset of stimulation (Enero & Langer, 1973). The radioactivity in the bathing solution and in the tissue was measured by scintillation counting as described by Adler-Graschinsky *et al.* (1972).

In separate experiments, the accelerans nerves were stimulated for 20 s at different frequencies at supramaximal voltage, and the maximal increase in atrial rate was determined. The interval between each period of nerve stimulation at different frequencies was 5 min, this being sufficient for the atrial rate to return to the resting values. The following frequencies of stimulation were applied: 0.1, 0.2, 0.4, 0.8, 1 6, 3 2, 6.4, 12.5, 25 and 50 Hz. Two consecutive frequency-response curves were determined in each preparation, the second starting 30 min after the end of the first.

Cumulative concentration-response curves to noradrenaline were determined once only in each of several preparations by stepwise increase in the concentration by a factor of three as soon as the response to the previous administration had levelled off (i.e at intervals of 2 to 4 minutes).

For studies of uptake and retention of [^{3}H]-noradrenaline, the atria were dissected as described above, placed in a separate beaker containing 5 ml of Locke solution, set in a Dubnoff metabolic incubator at 37°C and gassed with O_2. The drugs tested were added to the Locke solution which was then pre-incubated for 30 minutes. Then 20 ng/ml of [^{3}H]-noradrenaline was added and incubation was continued for a further 20 min after which the tissue was washed for 5 min in fresh Locke solution, blotted, weighed and homogenized with 5 ml of cold 0.4N perchloric acid containing EDTA 1.0 mg/ml and Na_2SO_3 1.25 mg/ml. Tritiated and cold noradrenaline were isolated by chromatography in alumina columns (Graefe, Stefano & Langer, 1973). Endogenous noradrenaline was determined according to Laverty & Taylor (1968) and [^{3}H]-noradrenaline was measured by scintillation counting.

Isolated cat nictitating membranes

Cats of 1.8 to 3.6 kg body weight and of either sex were used. The animals were anaesthetized with sodium pentobarbitone (35 mg/kg i.p.) and the trachea was cannulated. The eyeball was excised and the nictitating membrane with all the adjoining tissue was removed from the orbit. The tissue was placed in a Petri dish with modified Krebs solution previously equilibrated with 95% O_2 and 5% CO_2. The composition of the Krebs solution was as follows (mM): NaCl, 118.0; KCl, 4.7; $CaCl_2$, 2.6; $MgCl_2$, 1.2; NaH_2PO_4, 1.0; $NaHCO_3$, 25.0; glucose, 11.1; EDTA, 0.004 and ascorbic acid, 0.11. Under a binocular microscope, the medial muscle was dissected together with the postganglionic sympathetic fibres arising from the infratrochlear nerve which innervates this muscle of the nictitating membrane (Thompson, 1958). The cartilage on which the fibres of the medial muscle are inserted was fixed to the bottom of a 10 ml organ bath. The upper end of the muscle was connected to a force-displacement transducer and the tension developed by the muscle was recorded with a Grass polygraph. The temperature was maintained at 37°C and the organ bath was bubbled with 95% O_2 and 5% CO_2. The

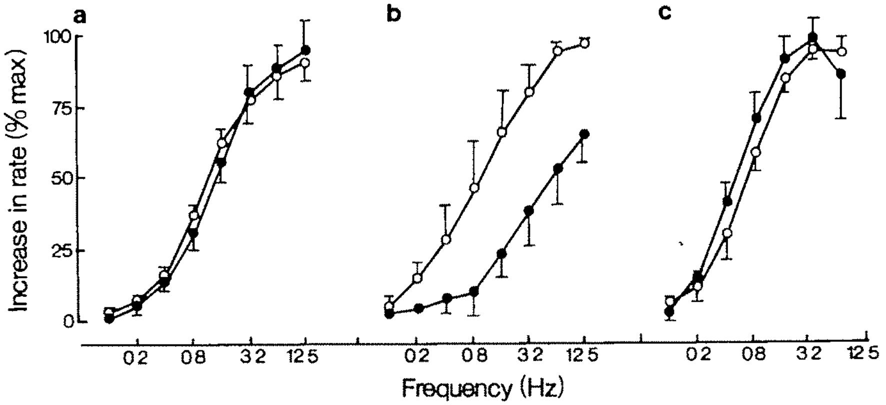

Figure 1 Effects of propranolol and of phentolamine on the positive chronotropic effects elicited by accelerans nerve stimulation in guinea-pig isolated atria. Ordinates: increase in atrial rate as % of the maximum of the first (before treatment) frequency-response curve. Abscissae: frequency of nerve stimulation (Hz). Frequency response curves obtained before (○) and 30 min after (●) treatment in (a), controls (*n* = 6); (b), atria exposed to propranolol (1.0 x 10^{-7}M) for 30 min before the second frequency-response curve (*n* = 3) and (c), atria exposed to phentolamine (3.1 x 10^{-6}M) for 30 min before the second frequency-response curve (*n* = 5). Mean values are shown. Vertical bars indicate s.e. mean. *n* = number of experiments.

infratrochlear nerve was pulled through shielded bipolar platinum electrodes for stimulation with monophasic square pulses of 0.5 ms duration and supramaximal voltage. Before starting experiments a period of 60 min was allowed to elapse during which the resting tension of the muscle was repeatedly adjusted to 2.5 g.

The postganglionic sympathetic fibres were stimulated at the progressively increasing frequencies of 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.5, 25 and 50 Hz, the frequency being stepped up whenever the response of the nictitating membrane to the previous frequency of stimulation had levelled off. Two frequency-response curves were determined in each preparation, the interval between them being 40 minutes. Twenty min after the second determination, a concentration-response curve to (–)-noradrenaline was determined by adding the drug cumulatively in such a way that the final concentration in the bath was increased by a factor of about 3 whenever a steady response to the previous concentration was achieved.

Statistical calculations were performed according to conventional procedures (Snedecor & Cochran, 1967).

The following drugs were used: (–)-noradrenaline bitartrate monohydrate, (±)-propranolol hydrochloride, (±)-isoprenaline hydrochloride, [*N*-2-(2,6-dimethylphenoxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (β-methyl-TM 10) and phentolamine hydrochloride.

Results

Chronotropic responses to accelerans nerve stimulation and to exogenous noradrenaline

The frequency-response curve for the positive chronotropic effects of accelerans nerve stimulation is shown in Figure 1. The maximum increase in atrial rate (112 ± 21 beats/min) was usually obtained with a frequency of stimulation of 12.5 Hz and this did not differ significantly from that obtained with exogenous noradrenaline (130 ± 5 beats/minute)

There was no difference between two consecutive frequency-response curves under control conditions (Figure 1a). In the presence of propranolol (1.0 x 10^{-7}M) there was a marked shift to the right (Fig. 1b) and after exposure to phentolamine, (3.1 x 10^{-6}M) the curve tended to shift to the left but not to a significant degree (Figure 1c). In the presence of propranolol, there was a similar shift to the right in the concentration-response curve to exogenous noradrenaline (Figure 2).

Transmitter overflow elicited by accelerans nerve stimulation

In a control group of atria there was no difference either between the chronotropic responses or between the fractional releases per shock observed with two consecutive periods of nerve stimulation

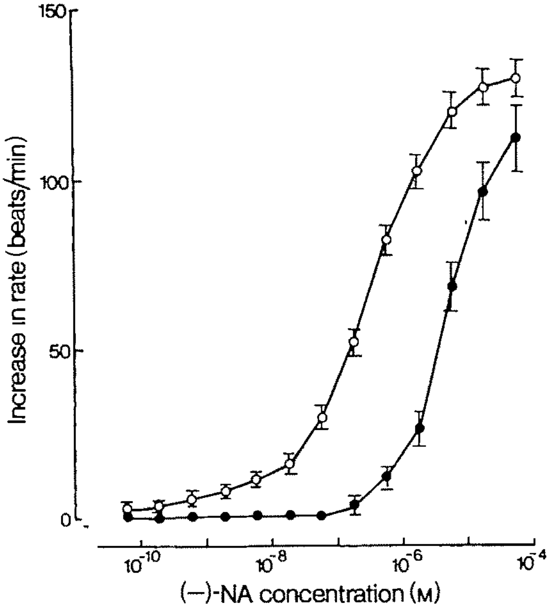

Figure 2 Effect of propranolol on the concentration-response curve to noradrenaline (NA) in spontaneously beating guinea-pig atria. (○) Controls (n = 16); (●) after exposure to propranolol (1.0×10^{-7}M) for 30 min (n = 7). Mean values are shown. Vertical bars indicate s.e. mean. n = number of experiments.

(Table 1). When isoprenaline (1.2×10^{-8}M) was added, a sustained positive chronotropic effect was obtained, the maximum being 98 ± 11 beats/min (mean ± s.e. mean of 6 observations). The increase in rate was still 84 ± 12 beats/min 20 min later when a second period of nerve stimulation was applied. The responses to nerve stimulation in the presence of isoprenaline were significantly reduced and this is attributable to the high atrial rate prior to nerve stimulation. Table 1 also shows that exposure to isoprenaline increased significantly the release of [^{3}H]-noradrenaline by nerve stimulation.

Propranolol, (1.0×10^{-7}M) did not affect the spontaneous rate but it decreased significantly the chronotropic responses to nerve stimulation. In the presence of the β-adrenoceptor blocking agent there was a significant reduction in the transmitter released by nerve stimulation as judged by the S_2/S_1 ratio (Table 1).

Exposure to phentolamine (3.1×10^{-6}M) reduced the spontaneous rate slightly but did not affect the positive chronotropic effect elicited by nerve stimulation. This concentration of the α-adrenoceptor blocking agent increased by more than three-fold the transmitter released by nerve stimulation (Table 1).

Effects of isoprenaline, propranolol and phentolamine on neuronal uptake of [^{3}H]-noradrenaline

The changes in transmitter overflow elicited by nerve stimulation in the presence of isoprenaline,

Table 1 Effects of isoprenaline, propranolol and phentolamine on atrial rate and on [^{3}H]-noradrenaline release elicited by nerve stimulation in guinea-pig isolated atria.

Experimental group	*No.*	*Resting rate (a) (beats/min)*	*Maximum increase in rate during stimulation (b) (beats/min)*	*Fraction released per shock (x 10^{-5}) (c)*	*Release S_2/S_1*
Control	6	R_1 = 180 ± 9	S_1 = 82 ± 8	S_1 = 1.11 ± 0.18	
		R_2 = 187 ± 10	S_2 = 72 ± 8	S_2 = 1.12 ± 0.22	1 03 ± 0.11
Isoprenaline	6	R_1 = 193 ± 9	S_1 = 100 ± 9	S_1 = 1 07 ± 0.24	
1.2×10^{-8}M		R_2 = 276 ± 12***	S_2 = 25 ± 14***	S_2 = 1.66 ± 0.39	1.55 ± 0 19*
Propranolol	10	R_1 = 187 ± 7	S_1 = 92 ± 9	S_1 = 0.93 ± 0.13	
$1 0 \times 10^{-7}$M		R_2 = 187 ± 7	S_2 = 66 ± 7*	S_2 = 0.55 ± 0.10	0.59 ± 0.08**
Phentolamine	4	R_1 = 202 ± 6	S_1 =. 80 ± 4	S_1 = 0.99 ± 0.23	
3.1×10^{-6}M		R_2 = 184 ± 4*	S_2 = 86 ± 4	S_2 = 3.02 ± 0.60	3.68 ± 1.13**

Mean values ± s.e. mean for: (a) Resting rates (R_1 and R_2) prior to each of two 1 min periods of nerve stimulation at 4 Hz (S_1 and S_2), (b) Maximum increase in atrial rate elicited by the first (S_1) and the second (S_2) period of nerve stimulation, (c) Fraction released per shock: total nCi released per shock divided by the total nCi remaining in the tissue at the onset of nerve stimulation. Isoprenaline, propranolol and phentolamine were added at 20, 40 and 30 min respectively before S_2. Significance of difference from the corresponding control value — *$P < 0.05$; **$P < 0.025$; ***$P < 0.005$.

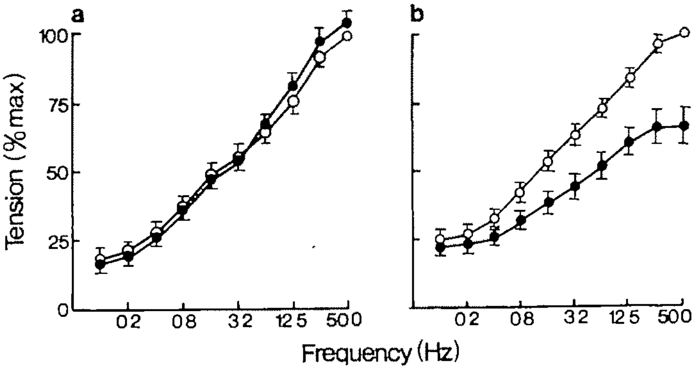

Figure 3 Effects of propranolol and of β-methyl-TM 10 on responses of the isolated medial muscle of the cat nictitating membrane to nerve stimulation. The relationship between the frequency of nerve stimulation and the percent of maximum response was determined before (○) and 30 min after (●) exposure to propranolol (1.0 x 10^{-7}M) in 6 preparations (a) and to β-methyl-TM 10 (3.9 x 10^{-6}M) in 7 preparations (b). Mean values are shown Vertical bars indicate s.e mean.

propranolol or phentolamine could be due to an effect of these drugs on neuronal uptake of noradrenaline This possibility was explored in experiments in which the uptake and retention of [^{3}H]-noradrenaline was determined after exposure to these drugs.

Table 2 shows that there was no difference between the endogenous noradrenaline contents of the groups studied. Exposure to isoprenaline, propranolol or phentolamine in the concentrations at which these drugs modified transmitter overflow during nerve stimulation, did not affect the accumulation of [^{3}H]-noradrenaline in the atria. Consequently, the effects of these drugs on transmitter release were not related to changes in neuronal uptake of noradrenaline.

Effects of propranolol or β-methyl-TM 10 on responses of the isolated nictitating membrane of the cat to nerve stimulation and to exogenous noradrenaline

It has been reported that propranolol reduces the responses to nerve stimulation by causing adrenergic neurone blockade (Mylecharane & Raper, 1970, Eliash & Weinstock, 1971). Consequently, it was of interest to determine whether the concentration of propranolol employed in our experiments affected responses to sympathetic nerve stimulation in the isolated nerve muscle preparation of the cat nictitating membrane.

There was no difference between the two consecutive frequency-response curves either in controls (n = 9) or in preparations treated with propranolol (1.0 x 10^{-7}M) (Figure 3a). In contrast, exposure to the adrenergic neurone blocking agent β-methyl-TM 10 (3.9 x 10^{-6}M) decreased the slope of the frequency-response curve to nerve stimulation producing a shift to the right and a depression of the maximum (Figure 3b). At these concentrations, neither β-methyl-TM 10 (7 preparations), nor propranolol (6 preparations) modified the pD$_2$ values or the maximum development of tension in response to exogenous noradrenaline by comparison with controls (35 preparations).

Table 2 Effects of isoprenaline, propranolol and phentolamine on neuronal uptake of [^{3}H]-noradrenaline in guinea-pig isolated atria.

Experimental group	n	*Endogenous content (μg NA/g tissue)*	*Neuronal uptake (ng [^{3}H]-NA/μg NA) (a)*
Controls	21	3.91 ± 0.20	31.3 ± 1.48
Isoprenaline 1.2 x 10^{-8}M	12	3.72 ± 0.18	27.3 ± 1.52
Propranolol 1.0 x 10^{-7}M	6	3.80 ± 0.20	33.0 ± 2.18
Phentolamine 3.1 x 10^{-6}M	6	3.16 ± 0.25	35.8 ± 2.45

(a) Neuronal uptake is expressed as ng [^{3}H]-noradrenaline (NA) taken up per μg of endogenous noradrenaline. The atria were exposed to the drug during a 30 min preincubation period and during a further 20 min incubation with 20 ng of [^{3}H]-noradrenaline. Values are mean ± s.e. mean. n = number of experiments.

Discussion

The frequency of stimulation at which the release experiments (Table 1) were carried out (4 Hz, 60 s) elicits a chronotropic response of approximately 80% of the maximum (Figure 1). Under these experimental conditions, exposure to phentolamine, in a concentration which does not inhibit neuronal uptake of $[^3H]$-noradrenaline (Table 2) increased more than three-fold the transmitter released by nerve stimulation. This effect is attributed to the block of the presynaptic α-adrenoceptors which mediate the negative feed-back mechanism that regulates noradrenaline release by nerve stimulation (Langer, 1974).

In spite of the increase in transmitter overflow observed in the presence of phentolamine the positive chronotropic response obtained under these conditions was not appreciably enhanced. It is likely that the negative chronotropic effect of phentolamine offsets the effect of increase in transmitter release. A negative chronotropic effect was also observed with phentolamine in the perfused cat heart (Farah & Langer, 1974).

An increase in transmitter release was observed in the presence of isoprenaline but this was much less pronounced than that obtained with phentolamine. As was shown for phentolamine, the effect of isoprenaline could also be dissociated from inhibition of neuronal uptake of noradrenaline (Table 2). In addition, the effect of isoprenaline on transmitter release appears to be unrelated to the activation of β-adrenoceptors of the effector organ because this agent also increases the release of $[^3H]$-noradrenaline during nerve stimulation in the cat nictitating membrane (Enero & Langer, unpublished observations) where the responses of the organ are mediated through α-receptors.

The decrease in transmitter release observed in the presence of the concentration of propranolol used (1.0×10^{-7}M) appears to be unrelated to either an increase in neuronal uptake of noradrenaline (Table 2) or to an adrenergic neurone blocking effect of the drug (Figure 3) because this concentration did not affect uptake of $[^3H]$-noradrenaline by guinea-pig atria nor reduce responses to nerve stimulation of the cat nictitating membrane. In addition, the concentration of propranolol employed in our experiments produced a shift to the right of the frequency-response curve to nerve stimulation in isolated atria (Figure 1) which should be attributed predominantly to the blockade of β-adrenoceptors because a similar shift to the right was obtained in the concentration-response curve to exogenous noradrenaline (Figure 2).

A decrease in responses to adrenergic nerve stimulation has been described after the administration of propranolol in the guinea-pig vas deferens (Mylecharane & Raper, 1970) and in the cat nictitating membrane *in vivo* (Eliash & Weinstock, 1971). These effects were attributed to an adrenergic neurone blocking effect of propranolol. In addition, it has been reported that high concentrations of propranolol potentiate responses to adrenergic nerve stimulation (Eliash & Weinstock, 1971) or increase the output of noradrenaline by nerve stimulation (Werner, Wagner & Schümann, 1971). The latter is related to the ability of high concentrations of propranolol to inhibit reuptake of noradrenaline (Werner *et al*, 1971).

The concentration of propranolol employed in our experiments did not reduce responses to nerve stimulation in the nictitating membrane and it reduced to nearly the same extent the responses to exogenous noradrenaline and to nerve stimulation in guinea-pig atria. Consequently, it is suggested that under our experimental conditions the effects of propranolol were not due to adrenergic neurone blockade.

The increase in transmitter release observed in the presence of isoprenaline and the decrease in transmitter release obtained during exposure to the β-receptor blocking agent, propranolol, indicate that these agents may influence transmitter release by acting on β-adrenoceptors. The results obtained are compatible with the existance in adrenergic nerve endings of a regulatory mechanism for noradrenaline release by nerve stimulation mediated through β-adrenoceptors. It is supposed that activation of this mechanism by β-adrenoceptor agonists leads to an increase in transmitter release during nerve stimulation.

According to our working hypothesis, the positive feed-back mechanism mediated via presynaptic β-adrenoceptors is activated during noradrenaline release until the transmitter in the synaptic gap reaches the threshold concentration that triggers the negative feed-back mechanism mediated by presynaptic α-receptors, resulting in inhibition of release. Thus, noradrenaline release by nerve stimulation may be modulated by two different presynaptic mechanisms. The first one, mediated by β-adrenoceptors, would be activated by low concentrations of the transmitter, leading to an increase in the release of noradrenaline per stimulus. The second one, mediated via α-receptors, would be triggered when higher concentrations of noradrenaline are reached in the synaptic gap, leading to the inhibition of transmitter release.

Compatible with this working hypothesis is the finding that the concentrations of noradrenaline

Table 3 Potencies of (−)-noradrenaline on the α- and β-adrenoceptors in several tissues

Tissue	*Species*	*Adrenoceptor*	*pD_2*	*Reference*
Splenic strip	cat	α	5.02*	Granata & Langer (1973)
Nictitating membrane	cat	α	5.04 ± 0.04	Langer & Rubio (1973)
Vas deferens	rat	α	5.23	Patil, LaPidus & Tye (1967)
Vas deferens	guinea-pig	α	4.48 ± 0.15	Nedergaard & Westermann (1968)
Tracheal strips	guinea-pig	β	6.52*	Furchgott (1967)
Atria	guinea-pig	β	6.52 ± 0.07	Langer & Rubio (1973)
Left atria	cat	β	7.64*	Osorio, Stefano & Langer (1974)
Papillary muscle	cat	β	7.66*	Kaumann (1970)
Fat cells (lipolysis)	rat	β	7.30*	Fain (1967)

pD_2: mean negative log of the molar concentration producing 50% of the maximal response.
* Calculated values.

required to activate postsynaptic α-adrenoceptors in general are at least 30 to 100 times higher than those necessary for the activation of the β-adrenoceptors (Table 3).

The results obtained in the present experiments do not exclude the possibility that propranolol and other β-adrenoceptor antagonists have an adrenergic neurone blocking effect under certain experimental conditions but in the experiments by Mylecharane & Raper (1970) and by Eliash & Weinstock (1971) blockade by propranolol of presynaptic β-adrenoceptors concerned with a positive feed-back mechanism may have contributed to the decrease in noradrenaline release by nerve stimulation.

The mechanism of action of β-adrenoceptor antagonists in the treatment of essential hypertension is not clear (Dorph & Binder, 1969, Prichard & Gillam, 1969; Tibblin & Åblad, 1969, Zacharias & Cowan, 1970; Thorpe, 1972). It is possible that a decrease in transmitter release obtained with propranolol contributes significantly to the hypotensive effects of this agent and other β-receptor blocking agents in hypertensive patients.

Several effects elicited by the stimulation of β-receptors have been attributed to the activation of adenyl-cyclase and the consequent increase in the tissue levels of cyclic adenosine-3′,5′-monophosphate (AMP). It is of interest that Wooten, Thoa, Kopin & Axelrod (1973) reported that dibutyryl cyclic AMP and theophylline increase the release of noradrenaline elicited by nerve stimulation in the guinea-pig vas deferens. In addition, monobutyryl cyclic AMP and several phosphodiesterase inhibitors increase transmitter release in the perfused cat spleen (Cubeddu, Weiner & Langer, unpublished observations). Consequently, it is possible that the proposed positive feed-back mechanism which is triggered by the activation of presynaptic β-adrenoceptors may be mediated through an increase in the levels of cyclic AMP in adrenergic nerve endings.

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INFLUENCE OF HISTAMINE H_1- AND H_2-RECEPTOR BLOCKERS ON SYMPATHETIC VASODILATOR AND VASOCONSTRICTOR RESPONSES IN CANINE PAW

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1 Vasodilator responses to histamine, bradykinin and sympathetic nerve stimulation were elicited in the perfused paw of dogs treated with bretylium (15-20 mg/kg) and atropine. The H_2-receptor blocking agent, burimamide, when administered in the dose of 5 mg/kg intravenously and 4 mg intra-arterially did not depress significantly these vasodilator responses. The subsequent administration of tripelennamine in the dose of 2.5-5 mg/kg intravenously and 4 mg intra-arterially produced a significant blockade of the response to histamine and reduced the sustained vasodilator response to nerve stimulation.

2 In guanethidine-treated dogs, tripelennamine administered in the same dose following burimamide produced a blockade of the respone to histamine comparable to that in the bretylium experiments, but decreased only the sustained vasodilator response to stimulation at 1 Hz. When the order of administration of the antihistamines was reversed in another group of guanethidine-treated dogs, tripelennamine had only a slight blocking effect on the response to histamine and did not affect the responses to nerve stimulation. Burimamide exerted a significant blocking effect on the response to histamine, but not to nerve stimulation. Another H_2-receptor blocking agent, metiamide, when given after tripelennamine, also had a marked blocking effect on the response to histamine and almost abolished the vasodilator response to 4-methylhistamine, an H_2-agonist. Nevertheless, even in the presence of this profound histamine blockade, the sustained vasodilator response to nerve stimulation remained unchanged.

3 In another group of experiments vasoconstrictor responses to exogenous noradrenaline and sympathetic stimulation were initially depressed by burimamide and later returned to control values. Tripelennamine increased these responses by its uptake blocking action.

4 It is concluded that the sustained vasodilator response is not antagonized by a specific antihistaminic action. The decrease in the sustained vasodilator response produced by antihistamines is probably attributable to potentiation of a residual adrenergic vasoconstrictor effect not completely blocked by bretylium.

Introduction

The introduction of burimamide (*N*-methyl-*N*′-4(-[4(5)-imidazolyl] butyl thiourea) has permitted an examination of certain actions of histamine which were previously resistant to blockade by antihistamines. This agent when given alone blocks the effects of histamine on gastric acid secretion and on the atrium, and when combined with mepyramine blocks the depressor action of histamine in the cat (Black, Duncan, Durant, Ganellin & Parsons, 1972). It was postulated by Black *et al.* (1972) that burimamide occupies histamine H_2-receptors to induce this blockade. H_2-receptors are believed to mediate vasodilatation and H_1-receptors vasoconstriction in the pulmonary vascular bed of the guinea-pig (Goadby & Phillips, 1973; Turker, 1973). The vasoconstrictor effect of histamine was reversed to a vasodilator response by the H_1-receptor blocking agent, mepyramine, and the vasodilatation was subsequently abolished by the H_2-receptor blocking agent, burimamide.

In previous studies it was reported that a vasodilator response of long duration was evoked by sympathetic nerve stimulation in the dog's paw or hind limb after adrenergic neuronal blockade or reserpine (Zimmerman, 1966; Beck, Pollard, Kayaalp & Weiner, 1966). This response has been designated sustained vasodilatation (Beck *et al.*, 1966). The response is predominantly mediated by a non-cholinergic transmitter since only the initial rapidly developing component is blocked by atropine (Beck *et al.*, 1966; Brody & Schaffer, 1970). Antihistamines reduce, but do not block sustained vasodilatation (Zimmerman, 1966, Rolewicz & Zimmerman, 1972). The inability to block this response may be attributable to the participation of H_2-receptors. If histamine is the mediator of sustained vasodilatation it might be assumed that the combined administration of an H_1- and H_2-blocker would be capable of abolishing the response. The present investigation was planned to determine the degree to which sustained vasodilatation elicited in the dog's paw by sympathetic stimulation is blocked by combined administration of an H_1-blocker, tripelennamine and an H_2-blocker, burimamide or metiamide. Also, since little is known about the effect of antihistamines on the sympathetic adrenergic system, especially with respect to the vasculature, their influence on vasoconstrictor responses to adrenergic nerve stimulation was also examined.

Methods

Dogs of either sex weighing 13.6-30.0 kg were anaesthetized by the intravenous administration of pentobarbitone sodium (30 mg/kg), and supplements of 30 mg were injected when necessary. Skeletal muscle relaxation was induced with decamethonium bromide (0.25 mg/kg), and this was supplemented at 30-60 min intervals with injections of 1 mg of the drug. All animals were artificially ventilated with a Harvard respirator. The left hind paw was prepared for perfusion as described by Zimmerman (1966). Briefly, this consisted of exposing the cranial tibial and lateral saphenous arteries in the paw and the left and right femoral arteries. Heparin sodium (7.5 mg/kg) was injected intravenously to prevent clotting. The paw was perfused by leading blood from the right femoral artery to a T 6S Sigmamotor pump which maintained flow constant through the catheterized cranial tibial artery. The left femoral and lateral saphenous arteries were ligated to isolate the arterial circulation of the paw. Systemic arterial and paw perfusion pressures were recorded on either a Gilson polygraph or Beckman dynograph. Since blood flow was constant, changes in perfusion pressure indicated changes in vascular resistance of the paw. Flow averaged 28.5 ± 0.7 ml/min (±s.e. mean) in the 26 experiments of this study. The left lumbar sympathetic nerve was stimulated with a Grass stimulator using pulses of 15-20 V, 1 ms in duration at 1, 3 and 10 Hz. The period of stimulation was in all cases 30 seconds. Vasodilator agonists were administered intra-arterially to the paw by bolus injections and antihistamines by slow infusions, intra-arterially and intravenously.

Sustained vasodilatation

At the beginning of the experiment the adrenergic vasoconstrictor response in the paw was elicited by sympathetic stimulation to insure function of the sympathetic innervation. Bretylium (15-20 mg/kg) was given acutely or guanethidine was administered on three consecutive days before the experiment to produce adrenergic blockade. Sympathetic nerve stimulation was carried out at 10 Hz for periods of 30 s until a maximal vasodilator response was attained. Atropine sulphate (0.25 mg/kg, i.v.) was administered and cholinergic blockade was determined by insuring that throughout the experiment the vasodilator response to 2 μg of acetylcholine injected intra-arterially was blocked. If acetylcholine produced significant vasodilatation, an additional dose of atropine (0.25 mg/kg) was given. Control vasodilator responses to three doses of histamine, two doses of bradykinin, and three frequencies of nerve stimulation were then elicited. In the experiments in which the H_2-receptor blocker, metiamide was administered, 4-methylhistamine, a selective H_2-agonist (Black *et al.*, 1972) was also given. Four groups of experiments were carried out. In one group ($n = 5$) bretylium was administered and the control vasodilator responses elicited. Burimamide was injected (5 mg/kg, i.v.) and infused (4 mg, i.a.) over a 10-15 min period and the vasodilator responses repeated once or twice after its administration. Tripelennamine was then administered (2.5-5 mg/kg, i.v.) and infused (4 mg/kg, i.a.) and the responses again repeated. A second group of experiments ($n = 6$) were similarly carried out in guanethidine-treated dogs. In a third group of experiments ($n = 5$), tripelennamine (2.5 mg/kg, i.v. and 4 mg, i.a.) was administered before burimamide, and then burimamide (8 mg, i.a.) was given. These experiments were carried out in guanethidine-treated dogs. Vasodilator responses were obtained after tripelennamine alone and then after burimamide. In this and the last group of experiments the sequence of eliciting the responses to histamine and nerve stimulation were alternated from one experiment to the other. In another group of guanethidine-treated dogs ($n = 6$) metiamide was infused intravenously at 0.25-5 mg kg^{-1} min^{-1}, after administration of tripelennamine.

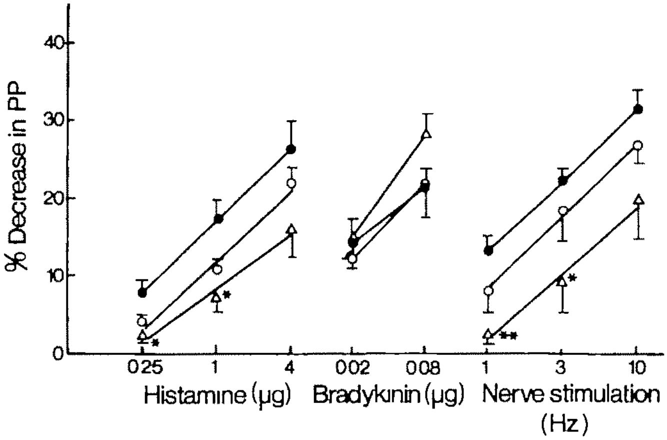

Figure 1 Dose-response curves to histamine and bradykinin, and frequency-response curves to nerve stimulation in (●) control period; (○) after burimamide (5 mg/kg, i.v. and 4 mg, i.a.) and (△) after tripelennamine (2.5-5 mg/kg, i.v. and 4 mg, i.a.). Values are mean of 5 experiments. Vertical bars show s.e. mean. $^*P < 0.05$, $^{**}P < 0.01$. PP = paw perfusion pressure.

Adrenergic vasoconstriction

Vasoconstrictor responses to three frequencies of sympathetic nerve stimulation and to three doses of noradrenaline injected intra-arterially were elicited in the control period and during an early and late period after burimamide alone, and after tripelennamine. In these experiments ($n = 4$) 5 mg/kg of burimamide and 2.5 mg/kg of tripelennamine were administered intravenously and 4 mg of each were given in addition intra-arterially.

Results are given as the mean with s.e. mean. For statistical analysis, the paired t test was used and differences were considered significant if $P < 0.05$. In order to minimize the influence of changes in perfusion pressure induced by the antihistamines on vasodilator responses, each response is expressed as a percentage of the control perfusion pressure just before the response.

Drugs

Histamine diphosphate (K and K Laboratories, Inc.); bradykinin (Nutritional Biochemicals), acetylcholine bromide (Eastman Organic Chemicals); atropine sulphate (K and K Laboratories, Inc.); noradrenaline bitartrate, as base (Winthrop Laboratories); 4-methylhistamine, burimamide and metiamide (Smith, Kline & French); bretylium tosylate (Burroughs Wellcome Co.); and tripelennamine and guanethidine, used as hydrochloride and sulphate salts (Ciba-Geigy Corp.). Burimamide and metiamide were solubilized with 1 or 2 M HCl and employed as the hydrochloride salts as recommended by Smith, Kline & French Labs.

Results

Effect of burimamide and tripelennamine on sustained vasodilatation

In this series of experiments bretylium (15-20 mg/kg) and atropine (0.25 mg/kg) were administered to block adrenergic vasoconstrictor and cholinergic vasodilator responses to sympathetic nerve stimulation, respectively. Vasodilator responses elicited in the paw by histamine, bradykinin and sympathetic nerve stimulation in the control period, after burimamide (5 mg/kg, i.v. and 4 mg, i.a.) and after both burimamide and tripelennamine (2.5-5 mg/kg, i.v. and 4 mg; i.a.) are shown in Figure 1. After the H_2-blocker (during the interval of 60-90 min following its administration), there were small decreases in the vasodilator responses to histamine and nerve stimulation, but no change in the response to the internal control, bradykinin. At earlier intervals some non-specific depression by burimamide was noted as evidenced by a reduction in the response to bradykinin. After the subsequent administration of tripelennamine, there were further decreases in the responses to histamine and nerve stimulation which were now statistically significant. No reduction was seen in the response to bradykinin. Tracings from a representative experiment demonstrating these effects are shown

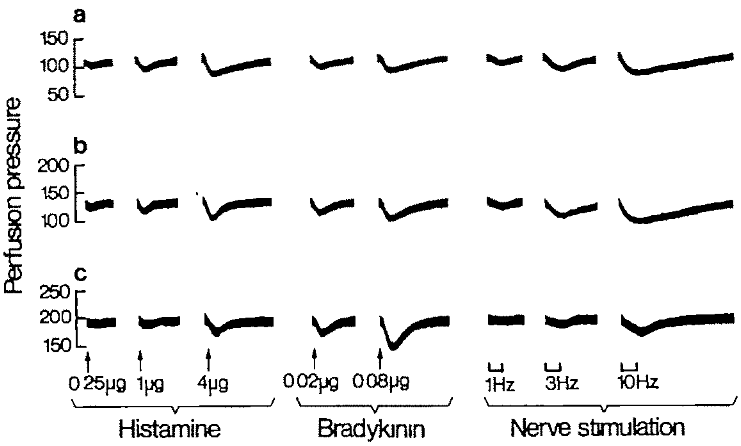

Figure 2 Responses of paw perfusion pressure (mmHg) to histamine, bradykinin and sympathetic nerve stimulation in (a) control period, (b) after burimamide (4 mg, i.a. and 5 mg/kg, i.v.) and (c) after tripelennamine (4 mg, i.a. and 5 mg/kg, i.v.). The responses were obtained during the period of 61 to 75 min after administration of burimamide and 18 to 30 min after tripelennamine. The period of sympathetic stimulation denoted by vertical lines is 30 seconds.

in Figure 2. Tripelennamine typically caused an increase in perfusion pressure and this vasoconstrictor effect tended to augment the absolute vasodilator responses. However, when the responses are expressed as a percentage of the control perfusion pressure, it is apparent that only the response to bradykinin remained the same, whereas responses to histamine and nerve stimulation were decreased.

Effect of burimamide or metiamide and tripelennamine on sustained vasodilatation in guanethidine-treated dog

In the experiments carried out in guanethidine-treated dogs, three doses of guanethidine, 5, 10 and 15 mg/kg (as the salt) were given intramuscularly in four and 2.5, 5 and 5-10 mg/kg doses (as base) were given intramuscularly or subcutaneously in 12 dogs. As adrenergic effects were abolished because of guanethidine treatment, a vasodilator response was obtained immediately upon sympathetic nerve stimulation in these animals, and there was less tendency for the response to increase with repetitive intervals of stimulation, as occurred after bretylium. Atropine (0.25 mg/kg) was given in these experiments to block the cholinergic component of the response. Control vasodilator responses, responses obtained after burimamide, and those obtained after burimamide and tripelennamine are shown in Figure 3. Burimamide in this dose caused some reduction in responses to histamine, bradykinin and nerve stimulation, but the changes were not significant. The subsequent administration of tripelennamine blocked the responses to histamine to a similar degree as in the experiments with bretylium (Figure 1), whereas only the response to nerve stimulation at 1 Hz was reduced significantly in contrast to the results obtained in bretylium-treated dogs.

In the second group of experiments carried out in guanethidine-treated animals the sequence of administration of antihistamines was reversed. This was done since the possibility existed that the effect of burimamide may have worn off before tripelennamine was given. The results presented in Fig. 4 demonstrate the effect of tripelennamine (2.5 mg/kg, i.v. and 4 mg, i.a.) given prior to the infusion of burimamide (8 mg, i.a.). Tripelennamine caused a small reduction in the response to histamine, but no decrease in the effect of nerve stimulation or bradykinin. The blockade of the response to histamine was of a lesser degree than that seen when burimamide had been given before tripelennamine (Figure 1), which indicates that the effect of the H_2-receptor blocker persisted in the first group of experiments. Burimamide administered after the H_1-receptor blocker caused a significant reduction in the responses to the lower doses of histamine during the period of 4.5-36 min after its administration. There was still evidence of histamine blockade at a later period (46-72 min) not shown in the figure. The responses to nerve stimulation and bradykinin were somewhat increased in this group of experiments after burimamide, but except for the response to

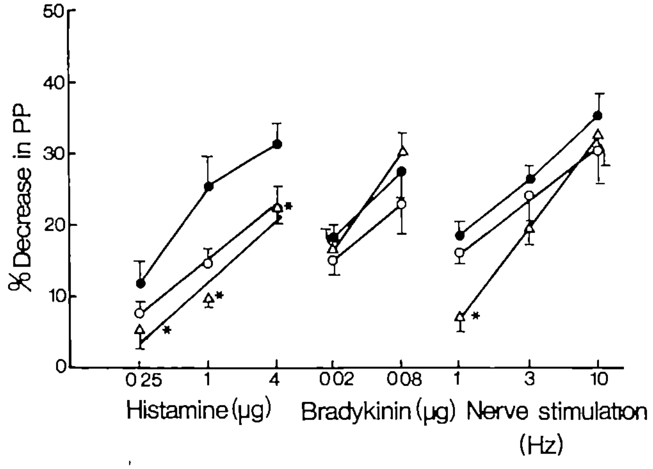

Figure 3 Dose-response and frequency-response curves in guanethidine-treated animals as in Figure 1. Values are mean of 6 experiments. Vertical bars show s.e. mean.

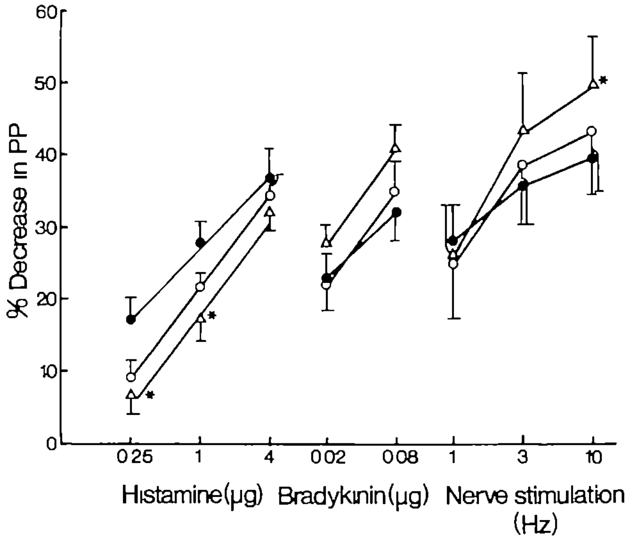

Figure 4 Dose-response and frequency-response curves in guanethidine-treated animals in (●) control period; (○) after tripelennamine (2.5 mg/kg, i.v. and 4 mg, i.a.) and (△) after burimamide (8 mg, i.a.). Values are mean of 5 experiments. Vertical bars show s.e. mean.

stimulation at 10 Hz were not significantly changed from the control.

The experiments in which metiamide (0.25-0.5 mg/kg) was infused continuously after the administration of tripelennamine were the most revealing with respect to the relative blockade of the responses to histamine and sustained vasodilatation. Responses to all three doses of histamine were markedly reduced and the responses to 4-methylhistamine were nearly abolished during infusion of metiamide (Figure 5). In contrast to this potent blocking effect on responses to histamine and methylhistamine, metiamide exerted no blocking action on the vasodilator responses to the internal control, bradykinin, or nerve stimulation. The latter responses tended to be somewhat larger in magnitude after tripelennamine and metiamide because of the vasoconstriction brought about by the antihistamines. Tripelennamine alone as in the

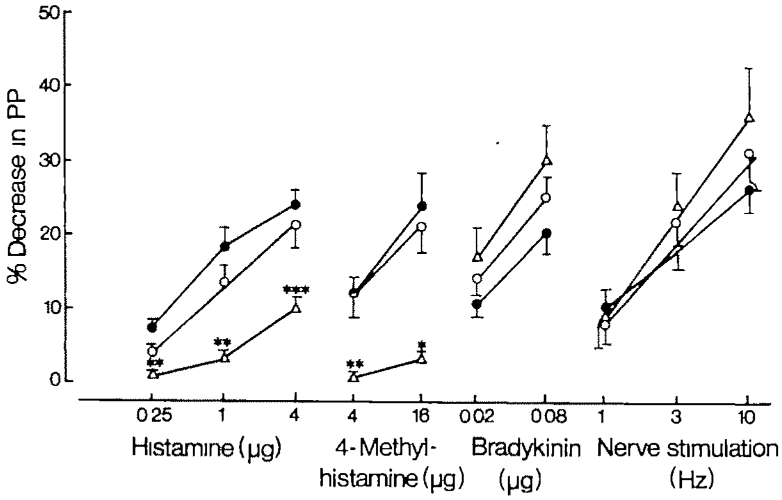

Figure 5 Dose-response curves to vasodilator agonists, including 4-methyl-histamine, and frequency-response curves obtained in guanethidine-treated animals in (●) control period; (○) after tripelennamine (2.5 mg/kg, i.v. and 4 mg, i.a.) and (△) during metiamide infusion (0.25-0.5 mg/kg, i.v.). Values are mean of 5 or 6 experiments. Vertical bars show s.e. mean. $^*P < 0.025$, $^{**}P < 0.01$, $^{***}P < 0.001$.

above described series of experiments had only a slight blocking effect on the response to histamine, and interestingly had no effect on the response to methylhistamine. The sustained vasodilator responses were similarly unaffected by tripelennamine when it was administered before an H_2-blocker in these guanethidine-treated dogs.

Table 1 shows the blood pressure and perfusion pressure values recorded in the control period, after burimamide, after metiamide and after tripelennamine for the four groups of experiments. Blood pressure remained stable and above 100 mmHg throughout these experiments. After an initial vasodilator response caused by the infusion of burimamide and to a lesser degree tripelennamine (not in Table), the perfusion pressure increased after administration of the antihistamines. Vasoconstriction was more pronounced after tripelennamine than following administration of burimamide or metiamide. Since release of catecholamines from adrenergic nerve endings does not occur after guanethidine, the vasoconstrictor action of tripelennamine is due either to a direct effect on vascular smooth muscle or to sensitization to the effect of circulating catecholamines released from the adrenal medulla.

Table 1 Blood pressure (BP) and perfusion pressure (PP) in control period, and after antihistamines in bretylium- and guanethidine (Guan) -treated animals

Blocker	*Period 1*	*BP*	*PP*	*Period 2*	*BP*	*PP*	*Period 2*	*BP*	*PP*
Bretylium (*n* = 5)	Control	140 ± 7	124 ± 14	Burim	138 ± 12	118 ± 8	Trip	137 ± 10	172 ± 9
Guan (*n* = 6)	Control	124 ± 4	109 ± 16	Burim	113 ± 6	122 ± 21	Trip	116 ± 9	164 ± 20
Guan (*n* = 5)	Control	114 ± 2	85 ± 16	Trip	127 ± 5	114 ± 14	Burim	117 ± 4	134 ± 11
Guan (*n* = 5 or 6)	Control *n* = 6	116 ± 9	94 ± 14	Trip *n* = 5	126 ± 12	143 ± 17	Met *n* = 5	135 ± 8	170 ± 14

Burim = burimamide, Trip = tripelennamine, Met = metiamide.

Table 2 Responses to noradrenaline (NA) and nerve stimulation (NS) in control period, after burimamide and after tripelennamine ($n = 4$)

		Control	*Burimamide (5 mg/kg, i.v. 4 mg, i.a.)*		*Tripelennamine (2.5 mg/kg, i.v. 4 mg, i.a.)*	
			Early	*Late*	*Early*	*Late*
NA	0.2 µg	62 ± 5	12 ± 3**	42 ± 7	65 ± 15	92 ± 16
	0.4	94 ± 8	21 ± 4***	76 ± 16	102 ± 18	162 ± 49
	0.8	144 ± 25	41 ± 10*	133 ± 44	186 ± 56	292 ± 125
NS	1 Hz	121 ± 20	48 ± 4*	100 ± 20	99 ± 20	125 ± 23
	3	160 ± 27	113 ± 16	174 ± 23	171 ± 28	214 ± 17*
	10	228 ± 31	196 ± 23	242 ± 21	303 ± 24	366 ± 55**

Values are mmHg, mean with s.e. mean. *$P < 0.05$, compared to control, **$P < 0.01$, compared to control, ***$P < 0.001$.

Effect of burimamide and tripelennamine on adrenergic vasoconstrictor responses

Vasoconstrictor responses to three doses of noradrenaline administered intra-arterially and three frequencies of nerve stimulation in the control period, after burimamide (5 mg/kg, i.v. and 4 mg, i.a.) and after the subsequent administration of tripelennamine (2.5 mg/kg, i.v. and 4 mg, i.a.) are given in Table 2. There was a marked decrease in the responses to noradrenaline and nerve stimulation early (2-16 min) after burimamide. The responses eventually returned to the control level during a later period following burimamide (87-101 minutes). Tripelennamine brought about potentiation of the responses to noradrenaline and nerve stimulation in the early period (7-35 minutes). This effect was even greater and statistically significant for the responses to 3 and 10 Hz during the later period (60-85 min) after tripelennamine.

Discussion

Administration of either the H_1-receptor blocking agent, tripelennamine or the H_2-receptor blocking agent, burimamide alone caused only a small decrease in the vasodilator response to histamine in the cutaneous vasculature of the dog's paw. Burimamide exhibited a non-specific depressant effect immediately after its administration, which was most apparent when it was given in a high dose before tripelennamine. This depressant effect was avoided by employing only the intra-arterial dose of 8 mg of burimamide or alternatively by the continuous intravenous infusion of metiamide. The presence of H_2-receptors in the paw vasculature was clearly demonstrated in these experiments by the marked reduction in the response to histamine and almost complete abolition of the response to 4-methylhistamine during infusion of metiamide following tripelennamine. Burimamide also caused significant blockade of the response to histamine after tripelennamine; however, the antagonism was not as complete as that produced by metiamide after tripelennamine. Stimulation of H_2-receptors by histamine does not completely account for the vasodilator response to this agonist in the paw. When tripelennamine was administered subsequent to burimamide, it too produced a very significant histamine blockade which suggests that H_1-receptors are also present. The weak blocking effect on the response to histamine in the paw of either burimamide or tripelennamine when given alone is similar to the finding made in the cat by Black *et al.* (1972) when examining the blood pressure response to histamine. In that study when mepyramine, an H_1-blocker, was administered before burimamide, the subsequent administration of the H_2-blocker was effective in antagonizing the depressor response to histamine. When given alone, burimamide had a negligible blocking effect.

Large doses of guanethidine were employed in the present investigation to produce a complete adrenergic blockade. The sustained vasodilator response obtained in the guanethidine-treated dog was not consistently reduced by either antihistamine or by their combined administration. The response to stimulation at 1 Hz was depressed by tripelennamine in one group of guanethidine-treated animals, but this may represent a nonspecific effect. The results obtained in the animals given bretylium differed from those obtained when guanethidine was employed. A substantial reduction in the sustained vasodilator response was found after combined administration of the H_1- and H_2-receptor blockers in the animals in which bretylium was used to produce adrenergic blockade. We attribute the apparent blockade of the sustained vasodilator response in the bretylium-treated animal to potentiation of a residual adrenergic vasoconstrictor effect by tripelennamine. Bretylium, unlike guanethidine, failed to eliminate the adrenergic response completely, and this was masked by the overriding vasodilation which occurs after bretylium. As shown in this study, tripelennamine is capable of potentiating the vasoconstrictor response to noradrenaline and adrenergic nerve stimulation, presumably by its blocking action on adrenergic uptake (Isaac & Goth, 1967). This effect may also have accounted for the reduction of the sustained vasodilator response by antihistamines that was observed in earlier investigations (Zimmerman, 1966; Rolewicz & Zimmerman, 1972). From the results of the present study it seems unlikely that histamine is the mediator of sustained vasodilatation in the dog's paw.

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A FURTHER INVESTIGATION INTO THE ENERGY DEPENDENCE OF ANGIOTENSIN II-INDUCED CONTRACTIONS OF ISOLATED SMOOTH MUSCLE PREPARATIONS

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1 The energy dependence of angiotensin and acetylcholine-induced contractions of rat descending colon and uterus was investigated.

2 Differences were observed in the effect of anaerobic substrate depletion upon responses of colon and oestrous and dioestrous uterus. These were attributed to differences in the energy metabolism of the tissues and were correlated with differences in tissue levels of glycogen.

3 The preferential reduction of angiotensin responses of dioestrous uterus and descending colon when exposed to 2,4-dinitrophenol, was evidence for an energy dependent stage in the angiotensin response distinct from the contraction process itself.

4 The absence of a preferential reduction of the angiotensin response of oestrous uterus when exposed to 2,4-dinitrophenol appeared to be related to the ability of this tissue to generate ATP by anaerobic glycolysis.

5 It was concluded that the energy for the angiotensin response may be derived either anaerobically or aerobically, depending upon the tissue.

Introduction

Angiotensin has been shown to produce a contraction of many smooth muscle preparations (Khairallah, 1971; Gross, 1971). In a previous report, we showed that the response of guinea-pig ileum to angiotensin appeared to involve an energy dependent step distinct from the contraction process (Crocker & Wilson, 1972; 1974). However, the response of this tissue to angiotensin is composed of a direct component and an indirect component through cholinergic nerves that can be blocked by atropine (Khairallah & Page, 1961; Robertson & Rubin, 1962; Godfraind, Kaba & Polster, 1966a, b). In the present study, we have extended these investigations to two smooth muscle preparations where the action of angiotensin has been shown to be wholly direct; rat descending colon (Regoli & Vane, 1964) and rat uterus (Khairallah & Page, 1961).

A preliminary account of some of these findings was communicated in September 1973 (Wilson, Crocker & Willavoys, 1974).

Methods

Preparations

White, Wistar rats (150-200 g) that had been starved for 12 h prior to experimentation were killed by cervical dislocation. Intestinal preparations were taken from the terminal 5 cm of colon immediately adjacent to the rectum and were flushed through with Tyrode solution, prior to mounting in 2.5 cm segments. Uterine preparations consisted of whole uterine horns removed from rats at oestrus or dioestrus, as determined by examination of the vaginal smear.

All preparations were suspended in 20 ml organ baths containing Tyrode solution bubbled with air. Colon segments were maintained at 32°C and uterine preparations at 28°C. The composition of the Tyrode solution was as follows: NaCl 137 (mM), KCl 2.7, $MgSO_4 . 7H_2O$ 1.1, $NaH_2PO_4 . 2H_2O$ 0.42, $NaHCO_3$ 11.9, $CaCl_2 . 2H_2O$ 1.8, glucose 5.6.

In experiments involving anoxia the Tyrode solution was placed under vacuum for 4 h and then the vacuum was replaced by oxygen-free nitrogen. The Tyrode reservoir and the organ bath were then gassed with nitrogen instead of air.

Recordings

It has been reported that isometric recording reveals more information about the individual components contributing to a muscle response than isotonic recording (Paton, 1961; Paton & Rothschild, 1965). When possible, therefore, we employed Devices 2ST02 isometric transducers with an applied resting tension of 1 g. However, uterine preparations deteriorated rapidly under isometric conditions and therefore these responses were recorded with Devices 2LDO1 isotonic transducers with an applied load of 1 g. Both isotonic and isometric transducers were coupled to Devices M2 recorders.

Tissues were equilibrated for 1.5 h and then the contractile responses were recorded at the maximal sustained deviation from the baseline. A tissue contact time of 90 s was employed for both angiotensin and acetylcholine. In all experiments, concentrations of acetylcholine and angiotensin were selected which produced responses approximately equal to the 50% maximal acetylcholine response.

Glycogen assay

The determination of the glycogen content of colon and uterine muscle samples was carried out with the method of Lo, Russel & Taylor (1970). The assay involved precipitation of tissue glycogen followed by spectrophotometric estimation using a modified phenolsulphuric acid technique and has been reported to be superior to the older anthrone method (Lo *et al.*, 1970). All reagents were of analytical grade and spectrophotometric measurements were made on a Pye Unicam SP500 MkII spectrophotometer.

Drugs and chemicals

The following chemicals were used: angiotensin II (Val 5-angiotensin II, Asp-β-amide; 'Hypertensin', Ciba), acetylcholine chloride (B.D.H.), 2,4-dinitrophenol (Sigma), oyster glycogen (B.D.H.), concentrated sulphuric acid (Analar), potassium hydroxide (Analar), sodium sulphate (Analar), crystalline phenol (Analar), ethyl alcohol, Absolute.

Calculation of results

All results have been expressed as a percentage reduction of the control response taken in normal Tyrode solution gassed with air. The control responses were measured when three successive exposures to the selected concentration of spasmogen had induced equal sized contractions. For each treatment, the reported results are the means of values obtained from at least six tissues. The effects of the experimental treatments have been analysed using Student's t test.

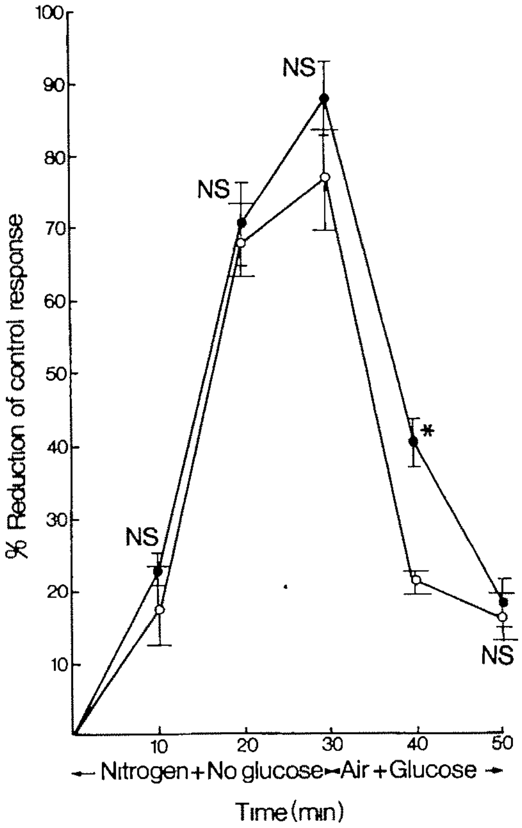

Figure 1 The effect of combined glucose removal and anoxia upon the contractile responses of rat descending colon to angiotensin and acetylcholine. (○) and (●) denote the mean percentage reduction (with s.e. mean) of the acetylcholine and angiotensin responses respectively. N.S. $P > 0.05$, $^*P < 0.01$.

Results

The effect of glucose lack and anoxia, separately and in combination

Exposure of preparations of rat descending colon and uterus to glucose-free Tyrode solution for periods of up to 2 h failed to affect their responses to either acetylcholine or angiotensin. Their responses to both agonists were also unaffected by exposure to nitrogen for periods of up to 3 hours. Since there was no effect with either treatment, the effect of combining the treatments was investigated.

Figure 1 shows the percentage reductions of angiotensin and acetylcholine responses which occurred during a 30 min exposure of rat

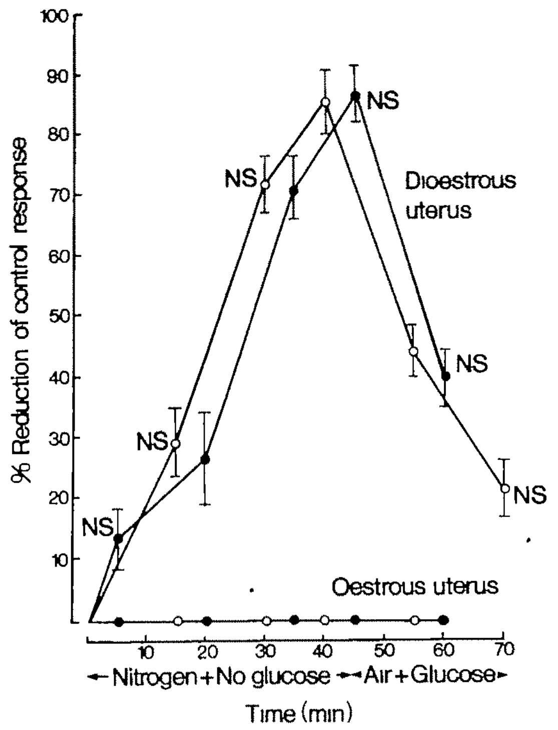

Figure 2 The effect of combined glucose removal and anoxia, upon the contractile responses of oestrous and dioestrous rat uterus to angiotensin and acetylcholine. (○) and (●) denote the mean percentage reduction (with s.e. mean) of the acetylcholine and angiotensin responses respectively. The percentage reduction of the angiotensin response is compared with that of the immediately preceding acetylcholine response. NS $P > 0.05$.

descending colon to glucose-free Tyrode and anoxia. There was a progressive reduction of responses to both agonists but at no time was there a significant difference between the percentage reduction of the angiotensin and acetylcholine responses. After 30 min the percentage reduction of the angiotensin response was 87.5 ± 4.9 ($n = 9$) and that of the acetylcholine response was 76.9 ± 8.1 ($n = 9$) which suggested a marked impairment of the contractile process.

Figure 2 shows the percentage reductions of the angiotensin and acetylcholine responses which occurred during a 45 min exposure of rat dioestrous uterus to glucose-free Tyrode solution and anoxia. With dioestrous uterus the results were similar to those obtained with descending colon (Figure 1). There was a progressive reduction of responses to both agonists with no significant difference between the percentages reduction of the angiotensin responses and the acetylcholine responses. After 40 min, acetylcholine responses were reduced by 85.8 ± 5.7% ($n = 6$) and after 45 min angiotensin responses were reduced by 86.9 ± 4.6% ($n = 6$). However, using the same conditions, the response of oestrous uterus to either angiotensin or acetylcholine was unaffected. Prolongation of exposure times for up to 5 h failed to affect the ability of this tissue to respond to either of the two agonists.

Measurement of tissue glycogen content

Although there was no difference in the responses of colon and uterus to acetylcholine compared with angiotensin during metabolic inhibition there was a difference between the responses of oestrous and dioestrous uterus which was significant for both angiotensin ($P < 0.001$) and acetylcholine ($P < 0.001$). Since under these conditions of metabolic inhibition, the tissue can derive energy from anaerobic glycolysis the difference in the responses of oestrous and dioestrous uterus might be due to differences in the levels of tissue glycogen.

The glycogen content of rat oestrous uterus, measured by the method of Lo *et al.* (1970), was 252.0 ± 5.0 mg/100 g wet weight of tissue ($n = 46$) which was significantly higher ($P < 0.001$) than the content of dioestrous uterus 147.0 ± 6.0 mg/100 g wet weight of tissue ($n = 30$). There was no significant difference between the glycogen content of descending colon taken from male rats, 115.0 ± 3.0 mg/100 g wet weight tissue ($n = 15$), from oestrous rats, 112.0 ± 3.0 mg/100 g wet weight of tissue ($n = 46$), or from dioestrous rats, 113.0 ± 2.0 mg/100 g wet weight of tissue ($n = 48$).

The effect of 2,4-dinitrophenol

To investigate further the importance of anaerobic glycolysis as an energy yielding process, experiments were performed using a Tyrode solution containing glucose (5.6 mM) as substrate and 2,4-dinitrophenol (0.1 mM). This concentration of 2,4-dinitrophenol has been shown to be effective in uncoupling oxidative phosphorylation in rat uterine tissue (Rangachari, Paton & Daniel, 1972).

Segments of descending colon from oestrous and dioestrous rats were exposed to this solution for 25 min and responses to acetylcholine and angiotensin were recorded. There was no difference in the responses obtained from dioestrous preparations compared with oestrous preparations but in both the percentage reduction

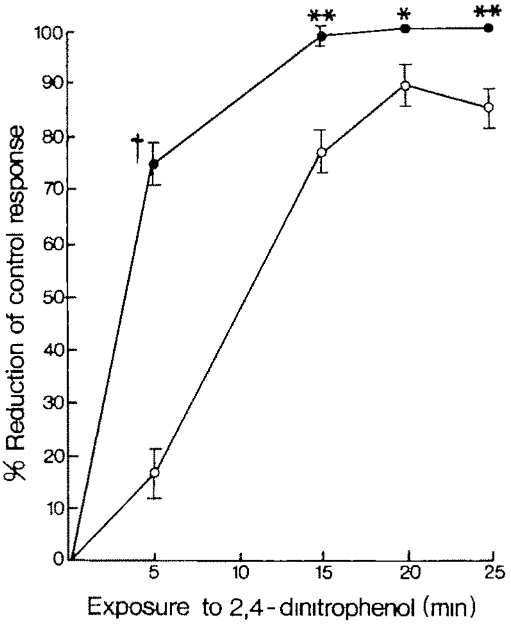

Figure 3 The effect of 2,4-dinitrophenol (0.1 mM) upon the contractile responses of dioestrous rat descending colon to angiotensin and acetylcholine. (○) and (●) denote the mean percentage reduction (with s.e. mean) of the acetylcholine and angiotensin responses respectively. * $P < 0.05$, ** $P < 0.01$, † $P < 0.001$.

of the angiotensin response was significantly greater ($P < 0.001$) than that of the acetylcholine response. Figure 3 shows the rapid increase in the percentage reductions of the responses to both agonists when dioestrous colon was exposed to 2,4-dinitrophenol (0.1 mM) for up to 25 minutes. After 5 min exposure to 2,4-dinitrophenol, the angiotensin response was reduced by 75.2 ± 4.1% ($n = 5$) and the acetylcholine response was reduced by 16.6 ± 4.8% ($n = 5$). The difference between the percentage reduction of the angiotensin response and that of the acetylcholine response was highly significant ($P < 0.001$). Although at 15 min both responses were markedly reduced and the angiotensin response almost abolished, there was still a significant difference ($P < 0.001$) between the percentage reduction of the angiotensin response (98.3 ± 1.6) and that of the acetylcholine response (77.4 ± 4.1). When a lower concentration of 2,4-dinitrophenol was used (0.05 mM) maximal inhibition occurred within 30 min of exposure and the angiotensin response was inhibited by 69.2 ± 2.2% ($n = 9$) and the acetylcholine response by 34.4 ± 4.3% ($n = 9$). The difference between the percentage reduction of the acetylcholine response compared with the angiotensin response was highly significant ($P < 0.001$).

Figure 4 shows the results obtained when

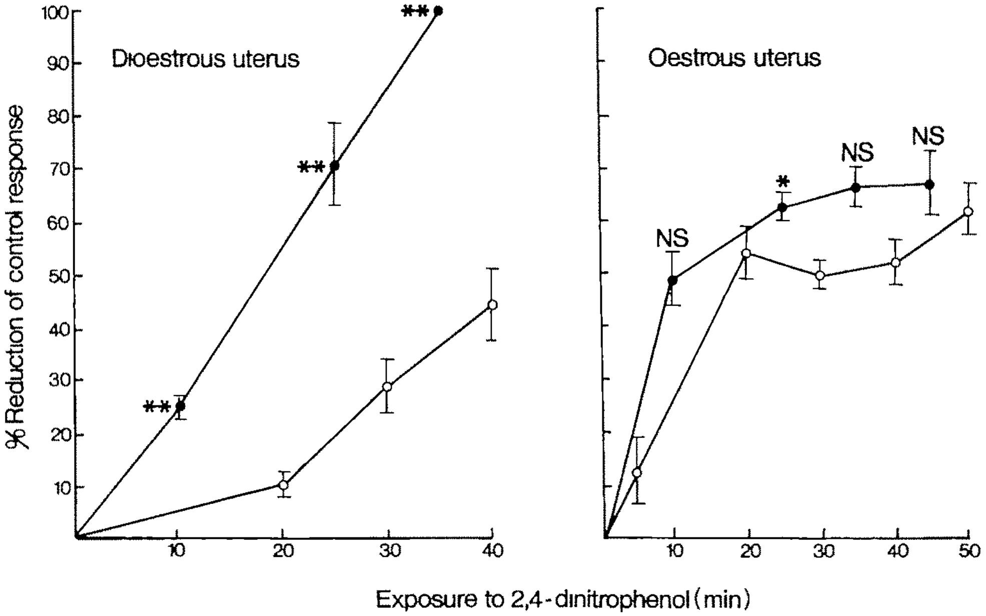

Figure 4 The effect of 2,4-dinitrophenol (0.1 mM) upon the contractile responses of rat oestrous and dioestrous uterus to angiotensin and acetylcholine. (○) and (●) denote the mean percentage reduction (with the standard error of the mean) of the acetylcholine and angiotensin responses respectively. The percentage reduction of the acetylcholine response is compared with that of the immediately preceding angiotensin response. * $P < 0.05$, ** $P < 0.001$.

oestrous and dioestrous uterus were exposed to 0.1 mM 2,4-dinitrophenol. The responses of dioestrous uterus to both angiotensin and acetylcholine were progressively and rapidly reduced during exposure to 2,4-dinitrophenol but at all times the reduction of the angiotensin response was significantly greater ($P < 0.001$) than that of the acetylcholine response. The rate of the reduction of the responses was slower in dioestrous uterus than in descending colon and complete abolition of the angiotensin response on dioestrous uterus was obtained after 35 min compared with 15 min for colon.

The responses of oestrous uterus to both agonists (Fig. 4) showed an initial rapid reduction followed by a slower reduction so that after 30 min exposure to 2,4-dinitrophenol (0.1 mM) the acetylcholine response was reduced by 50.0 ± 3.3% ($n = 5$) and the subsequent angiotensin response (at 35 min) was reduced by 65.7 ± 3.4% ($n = 5$). There was no consistent significant difference between the percentage reductions of the angiotensin and acetylcholine responses of oestrus during the period of exposure to 2,4-dinitrophenol (0.1 mM).

Discussion

In a previous study, we investigated the effect of various forms of metabolic inhibition upon responses of guinea-pig ileum to angiotensin and acetylcholine (Crocker & Wilson, 1972, 1974). It was shown that both substrate depletion and anoxia preferentially reduced responses induced by angiotensin and, on the basis of these findings, we proposed the existence of an energy dependent step in the angiotensin response distinct from the contractile process itself and associated with the direct component of angiotensin's action in this tissue. We proposed also that the energy for this step was provided from aerobic metabolism. However, the interpretation of these findings was complicated by the need to use a cholinoceptor antagonist to study the direct effect of angiotensin in guinea-pig ileum so in the present study we have investigated the energy dependence of the angiotensin response in tissues where angiotensin has no indirect, cholinergically, mediated action.

Anoxia and glucose lack separately failed to produce the differential reduction of the angiotensin response of rat colon that had been observed with guinea-pig ileum. Similarly, responses of rat dioestrous and oestrous uterus to angiotensin and acetylcholine were unaffected by anoxia or glucose lack separately. This failure to confirm our previous findings suggested that either our original hypothesis was incorrect or that differences in tissue metabolism made it difficult to demonstrate the same effects. This latter point is supported by many reports in the literature showing that tissues do vary in their susceptibility to metabolic inhibition. Other workers have also observed contractions of isolated smooth muscle preparations both under anaerobic conditions in the presence of glucose (Gross & Clark, 1923; Prasad, 1935; West, Hadden & Farah, 1951; Day & Vane, 1963; Furchgott, 1966; Shibita & Briggs, 1967; Hughes & Coret, 1968; Detar & Bohr, 1972) and under aerobic conditions in the absence of glucose (Prasad, 1935; Furchgott & Wales, 1951; Axelsson, Hogberg & Timms, 1965; Furchgott, 1966; Shibita & Briggs, 1967; Hughes & Coret, 1968; Coe, Detar & Bohr, 1968; Rangachari *et al.*, 1972). Presumably in our experiments enough energy was generated by anaerobic glycolysis of exogenous glucose to support the contractile responses to both agonists under conditions of anoxia or by aerobic metabolism of endogenous glycogen and β-oxidation of fatty acids after removal of glucose from the Tyrode solution.

When anoxia and glucose lack were combined differences were obtained between the responses of the three tissues to both agonists but again no differential reduction of the angiotensin response was observed. The responses of colon and dioestrous uterus to acetylcholine and angiotensin were reduced by the combined treatment whereas the responses of oestrous uterus were unaffected. Again these findings supported the idea that the problem of demonstrating a differential reduction of the angiotensin response might be related to differences in the metabolism of the tissues we were using. It has been shown that isolated smooth muscle preparations derive energy from carbohydrate metabolism and β-oxidation of fatty acids (Furchgott & Shorr, 1946; Coe *et al.*, 1968). It is also well known that concentrations of ATP and creatine phosphate are low in both uterine (Cspao & Gergely, 1950; Walaas & Walaas, 1950; Menkes & Csapo, 1952) and intestinal smooth muscle (Dworaczek & Barrenscheen, 1937; Born, 1956). Therefore, the ability of a preparation to respond under conditions of metabolic inhibition is a reflection of its ability to generate energy by the metabolic pathways operative under those conditions. It has been demonstrated by other workers that many isolated smooth muscle preparations are unable to maintain contractility under conditions of anaerobic substrate depletion (Gross & Clark, 1923; Garry, 1928; Prasad, 1935; West *et al.*, 1951; Furchgott, 1966; Shibita & Briggs, 1967; Rangachari *et al.*, 1972) when the tissue would be dependent upon anaerobic glycolysis of endogenous glycogen.

Therefore, in the present report, the marked effect of anaerobic substrate depletion on the responses of colon and dioestrous uterus compared with the lack of effect on the responses of oestrous uterus might be a reflection of differences in the energy produced by these tissues from anaerobic glycolysis of endogenous glycogen stores. Thus either oestrous uterus contained a larger amount of glycogen than dioestrous uterus or colon, or the glycolytic pathway was far more active in the oestrous uterus. However, both descending colon and dioestrous uterus were capable of generating sufficient energy to maintain contractions under anaerobic conditions in the presence of exogenous glucose. It seemed probable, therefore, that the activity of the anaerobic glycolytic pathway was not the limiting factor and that the differences in energy production under conditions of anoxia and glucose lack could be attributed to differences in the available glycogen stores.

An analysis of the glycogen content of rat uterus revealed that the glycogen level of oestrous uterus was significantly higher than that of dioestrous uterus. This confirmed several reports that uterine glycogen varied during the oestrous cycle (Boettiger, 1946, West, Jones & Loomis, 1953, West & Cervoni, 1955). The glycogen content of rat descending colon was found to be significantly lower than that of dioestrous uterus but was independent of either the stage of the oestrous cycle or the sex of the animal. Thus oestrous uterus which was unaffected by anaerobic substrate depletion contained significantly more glycogen than either dioestrous uterus or descending colon, both of which were inhibited by anaerobic substrate depletion. Further, descending colon contained significantly less glycogen than dioestrous uterus and was more rapidly inhibited by anaerobic substrate depletion.

Thus we had been able to demonstrate that the differences in the responses of these tissues after metabolic inhibition might indeed be related to differences in their metabolism which in turn might be the reason why we had not confirmed our previous findings (Crocker & Wilson, 1972; 1974). This is illustrated by our present observations that rat descending colon was insensitive to conditions of metabolic inhibition which had reduced the responses of guinea-pig ileum and revealed the differential effect on the angiotensin response. Our findings with colon are supported by reports from several workers that there is a metabolic gradient down the length of the intestine (Alvarez, 1922; Evans, 1923; Prasad, 1935; Dickens & Weil-Malherbe, 1941; Farah, West & Angel, 1950; Sherratt, 1968) and that colon preparations are far less dependent upon oxidative metabolism than preparations of the small intestine (Prasad, 1935; Farah *et al.*, 1935).

In the experiments where preparations were exposed to anoxia, nitrogen was substituted for the normal air supply to the tissues so that the degree of anoxia produced was liable to variation and was probably never complete because of the difficulty of removing all dissolved oxygen from the Tyrode solution. There have been several reports which demonstrated that the effect of anoxia was markedly dependent upon the actual reduction in P_{O_2} achieved (Detar & Bohr, 1968a, b, 1972; Namm & Zucker, 1973). To overcome this problem, a chemical inhibitor of oxidative metabolism, 2,4-dinitrophenol, was used.

2,4-Dinitrophenol has been extensively used to investigate the metabolism of smooth muscle preparations and its effects have been shown to be consistent with its known mode of action as an uncoupler of oxidative phosphorylation (Farah *et al.*, 1950; West *et al.*, 1951; Born & Bulbring, 1955; Daniel, 1964; Rangachari *et al.*, 1972; Greenberg, Wilson & Long, 1973). In our experiments we used 2,4-dinitrophenol in a Tyrode solution containing glucose as an exogenous substrate for glycolysis so that energy production would be dependent upon anaerobic glycolysis and the tissues should be capable of some degree of response to applied spasmogens (Daniel, 1964; Rangachari *et al.*, 1972; Greenberg *et al.*, 1973).

Dinitrophenol progressively reduced the responses of rat descending colon and dioestrous uterus to acetylcholine and angiotensin, but at all times the percentage reduction of the angiotensin response was significantly greater than that of the corresponding acetylcholine response. With oestrous uterus, however, there was a slow reduction of responses to both acetylcholine and angiotensin with no preferential reduction of the angiotensin response. This difference between the effect of dinitrophenol on oestrous uterus and that on dioestrous uterus and descending colon was consistent with the biochemical changes which occur in the uterus during the course of the oestrous cycle. Several workers have reported that anaerobic glycolysis is more efficient in oestrous uterus than in dioestrous uterus (Kerly, 1937; Kerly, 1940; Walaas, Walaas & Loken, 1952). In addition, it is known that the relative efficiencies of anaerobic and aerobic carbohydrate metabolism in uterine tissue varies during the oestrous cycle (Kerly, 1937). Anaerobic metabolism, the most important energy yielding process (West *et al.*, 1953), is at maximum efficiency in the early oestrous uterus when aerobic metabolism is at minimum efficiency. Inhibition of aerobic metabolism would therefore be expected to have less effect upon the overall capacity of oestrous uterus to generate energy than it would on dioestrous uterus. The capacity of the oestrous uterus to generate ATP anaerobically might then mask the differential reduction of the angiotensin response seen in dioestrous uterus and descending colon.

The reduction of the induced responses of the oestrous uterus by 2,4-dinitrophenol contrasted with the lack of effect produced by combined substrate depletion and anoxia. 2,4-Dinitrophenol was used in the presence of glucose and thus it would be expected that the reduction of responses would be less than the reduction produced by anaerobic substrate depletion, if the dinitrophenol solely acted as an uncoupling agent. There was the possibility that 2,4-dinitrophenol might also interfere with the combination of the agonists with their receptors but this seemed unlikely since we have shown that potassium-induced contractions of these tissues were affected by 2,4-dinitrophenol in the same way (Crocker & Wilson, unpublished observations). Also, other workers have reported an inhibitory action of 2,4-dinitrophenol upon anaerobic contractions of smooth muscle preparations, and this has been attributed to interference with the formation of energy rich phosphate bonds (Farah *et al.*, 1950; West *et al.*, 1951).

The preferential reduction of responses of dioestrous uterus and descending colon to angiotensin, produced by exposure to 2,4-dinitrophenol, indicated that the angiotensin response of these tissues was more dependent upon energy than the acetylcholine response. The primary action of acetylcholine on smooth muscle cells has been reported to involve an increase in the passive conductance of the membrane to sodium ions (Bolton, 1972) and therefore the energy requirement of an acetylcholine-induced contraction is that of the muscle contraction process itself. Thus, the greater impairment of the angiotensin response that was observed in the experiments with 2,4-dinitrophenol was indicative of the existence of an energy dependent stage in the angiotensin response, distinct from the actual contraction process.

This confirms the results that we reported for guinea-pig ileum (Crocker & Wilson, 1972, 1974) and provides further evidence that the energy dependent stage is involved in the direct action of angiotensin upon smooth muscle. In addition, however, it is evident that the energy supply for this stage of the angiotensin response is variable and dependent upon the tissue and the experimental conditions. In guinea-pig ileum the energy was supplied by aerobic metabolism, but in descending colon and rat uterus, energy may be supplied by aerobic and anaerobic metabolism.

The nature of this energy dependent stage is not yet resolved and further experiments are in progress to investigate a possible relationship between the action of angiotensin and active ion movement.

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EFFECTS OF ADRENALINE, NORADRENALINE, ISOPRENALINE AND SALBUTAMOL ON THE PRODUCTION AND RELEASE OF RENIN BY ISOLATED RENAL CORTICAL CELLS OF THE CAT

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1 Isolated renal cortical cells of the cat have been demonstrated to produce renin on incubation *in vitro*. After 2 h of incubation, without added agonist, the total amount of renin in the flask increased by a mean of 27.2%. The increase in renin content of the incubation flask was found to be present in the medium.

2 Noradrenaline (1.18×10^{-4} M) and adrenaline (1.09×10^{-4} M) added to the incubation medium stimulated renin production by 45 and 34% respectively, compared with the incubated controls. Most of the increase in renin production was present in the incubation medium.

3 Isoprenaline did not stimulate renin production. However, when added to the incubation medium at a concentration of 0.72×10^{-4} M there was a significant decrease in the cellular content and a significant increase in the medium content of renin. This increase was at least as great as that observed with adrenaline and noradrenaline.

4 Salbutamol had an effect similar to isoprenaline, i.e. it induced the release of renin into the medium without affecting production. In this respect it was about a third as potent as isoprenaline.

Introduction

A number of stimuli have been reported to alter the release of renin *in vivo*. These include changes in renal haemodynamics, changes in rate of sodium transport within the renal tubules, and the actions of catecholamines, either locally released or carried to the renin-containing cells via the circulation (cf. Vander, 1967; Davis, 1971). While the first two of these mechanisms are probably only amenable to study *in vivo*, the action of catecholamines can be studied on the isolated renal cortical cell. The technique would appear to be particularly useful in studying the direct effect of sympathomimetic substances, in isolation from other mechanisms of renin release which might be activated *in vivo* by these drugs. The method has been used by Michelakis, Caudle & Liddle (1969) in a study of the effect of adrenaline, noradrenaline and cyclic adenosine 3′,5′-monophosphate (AMP) on net renin production by dog isolated renal cortical cells *in vitro*. They observed that all of these substances stimulated renin production.

There is increasing evidence that the action of catecholamines in stimulating renin release is mediated by a β-adrenoceptor, as judged by the effect of infusion of isoprenaline (Ueda, Yasuda, Takabatake, Iizuka, Iizuka, Ihori & Sakamoto, 1970; Van Dongen, Peart & Boyd, 1973) and by blockade of the renin-releasing effect of catecholamines by propranolol (Assaykeen, Clayton, Goldfein & Ganong, 1970; Meurer, 1971, Passo, Assaykeen, Goldfein & Ganong, 1971; Coot, Johns, Macleod & Singer, 1972; Loeffler, Stockigt & Ganong, 1972). The nature of the β-adrenoceptor mediating renin release is not known, as most of the work has been done with propranolol, which is non-selective. Several recent studies in hypertensive subjects have indicated that the cardioselective drugs practolol (Esler & Nestel, 1973) and ICI 66082 (Aberg, 1974) also inhibit renin production although systematic comparisons of the relative effectiveness of these drugs in blocking renin release are not available. It would appear to be of considerable therapeutic interest if the characteristics of this β-adrenoceptor were available.

In the present study, the ability of isolated renal cortical cells of the cat to produce renin on incubation *in vitro* has been investigated and the effect of added adrenaline, noradrenaline, isoprenaline and salbutamol on the production and release of renin during incubation has been studied.

Methods

Experiments were performed on kidneys obtained from 14 male cats in the weight range 3.1-6.0 kg. One kidney was removed from each of 10 cats anaesthetized with sodium pentobarbitone (42 mg/kg i.p.). Four cats were anaesthetized with ethyl chloride/ether and these had both kidneys removed.

All glassware used in this study was siliconized (Repelcote, Hopkin & Williams), thoroughly rinsed in deionized water and sterilized overnight in an oven at 120° C.

Preparation of cortical cell suspension

Cortical cell suspensions were prepared by an adaptation of the method devised for use in the dog by Michelakis *et al.* (1969). Kidneys were removed through retroperitoneal incisions and immediately placed in ice. The renal artery was then cannulated with fine polythene tubing attached to a syringe needle, and the kidney flushed through with cold 0.9% w/v NaCl solution (saline) until the fluid emerging from the renal vein was clear (approximately 30 ml). The renal vein and ureter were clamped with a Spencer-Wells forceps and incubation medium forced through the cannula until visible in the subcapsular space (6-8 ml). The incubation medium was Minimum Essential Medium with Earle's Basic Salt Solution (Bio-cult, Glasgow), to each 100 ml of which was added 1.3 ml 200 mM glutamine (Bio-cult). This medium differs in composition from that used by Michelakis *et al* (1969) in that it contains calcium at a concentration of 1.7 mM. The Spinner modified medium used by these authors contained no calcium, which has since been reported to be a requirement for optimum renin release (Michelakis, 1971).

After decapsulation, the renal cortex was removed and chopped with a scalpel blade to yield pieces 1-3 mm in each dimension. These were then placed in 25 ml incubation medium containing 0.05% w/v collagenase (Collagenase Form II, Koch-Light) and slowly stirred with a teflon-coated magnetic stirrer at room temperature and bubbled with 95% O_2, 5% CO_2 for 75 minutes. This period of digestion was compatible with maximum tissue disaggregation, but minimal cell damage.

At the end of the digestion period the mixture was filtered through a double layer of gauze and the filtrate centrifuged at 125 *g* for 5 min at 4° C. The supernatant was removed and the cells resuspended in 5 ml fresh medium at 4° C. This procedure was repeated twice more to remove all traces of collagenase. The cells were then finally resuspended in 25 ml fresh medium if one kidney had been utilized or 35 ml when two kidneys had been prepared.

Experimental incubations

Aliquots (2.5 ml) of cell suspension were pipetted into 25 ml conical flasks with ground glass stoppers. Nine or 12 flasks were prepared, depending on the amount of suspension available. These were subdivided into groups of three. One group acted as a control and to each of the flasks in other groups one of the four drugs was added. One flask from each set of three was frozen immediately to serve as a non-incubated control, and the other two were incubated for 2 h at 37° C in a shaking water bath and bubbled with 95% O_2, 5% CO_2 at 20 min intervals.

At the end of the incubation, the contents of each flask were transferred to centrifuge tubes and centrifuged at approximately 2,300 *g* for 15 min at 4° C. After the supernatant had been removed, the cells were resuspended in 5 ml fresh medium at 4° C and homogenized with a motor-driven teflon pestle. Both supernatant and homogenate were stored at −10° C until the renin was assayed. A flow chart for incubation procedures is given in Figure 1.

Assay of renin

Renin activity was estimated according to the method of Coot *et al.* (1972) developed for the cat from an original method by Ryan, McKenzie & Lee (1968).

A 50 μl experimental sample was diluted with 4.95 ml saline; 0.5 ml of this mixture was added to 0.25 ml 40 mM disodium edetate and 0.25 ml Trasylol (FBA Pharmaceuticals). A 0.25 ml sample of this final mixture was incubated at 42° C with 0.5 ml renin substrate prepared from nephrectomized cats (Coot *et al.*, 1972), for between 40 and 48 hours. Preliminary tests indicated that angiotensinase activity was negligible under these conditions. This was confirmed for each batch of incubations.

Angiotension I generated during the incubation was assayed against standard 1-asp 5-isol angiotensin I (Schwarz Bioresearch) by the rat blood-pressure bioassay. Assay of the non-incubated controls ensured that there was no interference in the bioassay from the catecholamines added to the suspension. Because of high dilutions of the renal cell homogenate necessary for the renin assay, the catecholamine concentrations were reduced to sub-threshold levels in the bioassay.

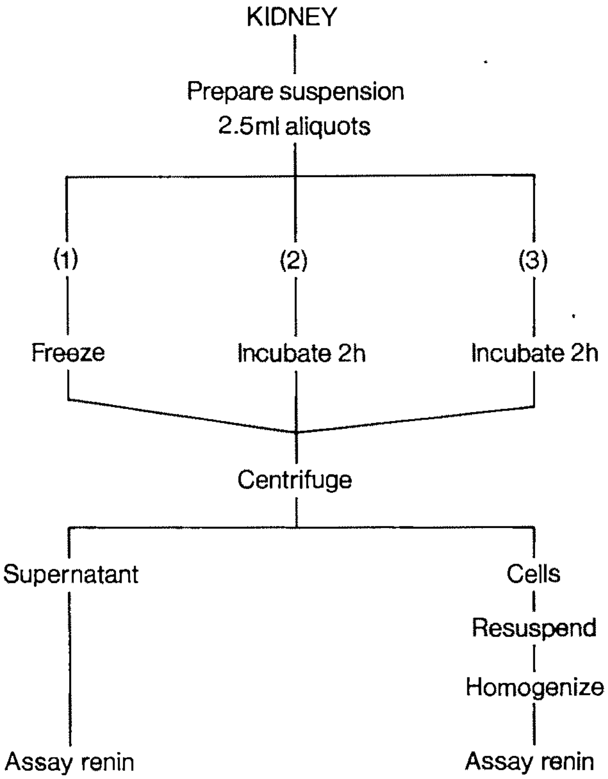

Figure 1 Procedure for the separation and incubation of renal cortical cells and preparation of the contents of the incubation flasks for renin assays.

Each sample was bracketed twice between doses of standard angiotensin I. Limits of the brackets were reduced so that the unknown could be measured at better than ± 10%.

Drugs

The following materials were used: noradrenaline (Levophed, Winthrop Laboratories); adrenaline (adrenaline tartrate, Evans Medical); isoprenaline (isoprenaline sulphate, Boots Ltd.); salbutamol (salbutamol sulphate, Allen & Hanbury, Ltd.).

Calculations

From the measured angiotensin I concentration, renin activity was expressed as ng angiotensin I generated per h incubation with substrate and per 2.5 ml suspension. The mean of the duplicates was calculated and both the incubated cell content and release were expressed as a percentage of the appropriate non-incubated control (cell content). Total renin was calculated by simple addition of the incubated cell content and release. Results were compared by Student's paired *t* test.

Results

Adrenaline and noradrenaline

These experiments were performed to indicate the sensitivity of the preparation and to confirm the action of these two catecholamines on renin secreting mechanisms in this species.

Preliminary experiments were carried out measuring total renin only, which indicated that renin production was dose-dependent at concentrations of adrenaline and noradrenaline between 0.44 and 1.90 x 10^{-4}M. For the present study, the doses chosen were 1.09 and 1.18 x 10^{-4}M as these were estimated to give 70% of the maximal response.

Table 1 gives the results obtained in eight experiments with noradrenaline and adrenaline respectively. The cell content of renin did not alter during incubation in the absence of added catecholamine nor was any significant difference observed in the presence of noradrenaline (+6.1%) or adrenaline (+1.9%) (Table 1).

Renin release increased significantly during incubation with noradrenaline. The amount

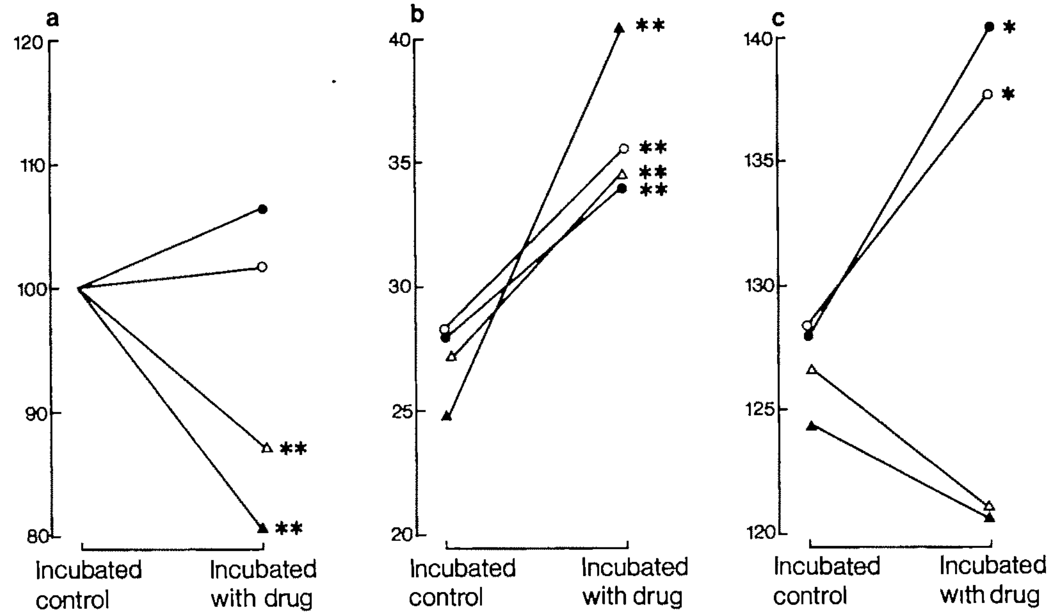

Figure 2 Effect of sympathomimetic amines on renin production and release *in vitro*, (a) cell content of renin (% of non-incubated control), (b) renin release (% of non-incubated control cell content), (c) total renin (content plus release) (●) Noradrenaline 1 18 × 10^{-4} M, (○) adrenaline 1.09 × 10^{-4} M; (▲) isoprenaline 0.72 × 10^{-4} M; (△) salbutamol 1.67 × 10^{-4} M. In all experiments $n = 8$. $^{*}P < 0\ 05$; $^{**}P < 0\ 01$.

released in control flasks was 27.8% (of non-incubated control values) compared with 34.3% in flasks containing noradrenaline ($P < 0.01$). A significant increase in renin release was also observed with adrenaline. The release rose from 28.3% in control flasks to 35.7% in adrenaline-containing flasks ($P < 0.01$).

When the results are expressed as the sum of cell content and the amount released, values increased from 127.8% (control) to 140.3% in the presence of noradrenaline ($P < 0.05$) and from 128.3 to 137.6% in the presence of adrenaline ($P < 0.05$).

Isoprenaline

No report has been published on the effect of isoprenaline on renal tissue *in vitro*, although it has

Table 1 Effect of noradrenaline, adrenaline, isoprenaline and salbutamol on renin production *in vitro*

Drug	*Content*		*Release*		*Total*	
	Incubated Control	*Test*	*Incubated Control*	*Test*	*Incubated Control*	*Test*
Noradrenaline (1.18 × 10^{-4}M)	100.0	106.1 ± 3 4	27.8 ± 4.6	34.3 ± 5.2**	127.8 ± 4.5	140 3 ± 6.2*
Adrenaline (1.09 × 10^{-4}M)	100.0	101.9 ± 3 1	28.3 ± 4.3	35.7 ± 4.7**	128.3 ± 4.3	137.6 ± 4.8*
Isoprenaline (0.72 × 10^{-4}M)	100.0	80 1 ± 4.5**	24.6 ± 2.95	40.5 ± 5.12**	124 6 ± 3.0	120.6 ± 3.9
Salbutamol (1.67 × 10^{-4} M)	100.0	86.1 ± 3.5**	26.9 ± 4.3	34.7 ± 4.7**	126.9 ± 4.3	120.8 ± 5.3

Results are expressed in terms of the cell content of renin in the unincubated control flasks which are designated as 100%. $n = 8$ for all drugs. Mean values with s.e. mean are given.
** Control vs drug $P < 0.01$; * Control vs drug $P < 0.05$ (Student's paired t test).

been reported to be effective in stimulating renin release *in vivo.* Table 1 shows the results of eight experiments. Isoprenaline, at a concentration of 0.72 x 10^{-4}M, is extremely potent in stimulating renin release. The control value was 24.6% compared with 40.5% in flasks containing isoprenaline ($P < 0.01$). The increased release seems to be at the expense of intracellular stores of renin. When isoprenaline was present during the incubation the cell content fell to 80.1% of the unincubated control value ($P < 0.01$). The total content of renin in the isoprenaline-containing flasks after incubation was 120.6% of the unincubated control value compared with 124.6% in control flasks. This decrease was not statistically significant.

Salbutamol

In order to derive more information on the nature of the β-adrenoceptor involved, the drug salbutamol, a selective β-adrenoceptor stimulant, was used. A preliminary dose-response curve showed salbutamol to be 2-3 times less potent than isoprenaline in stimulating renin release and so a concentration of 1.67 x 10^{-4}M was chosen to be approximately equipotent to the dose of isoprenaline used.

The results of eight experiments are presented in Table 1. It is apparent that although salbutamol is less potent than isoprenaline in stimulating renin release, the pattern of the response is similar to that obtained with isoprenaline. Cell content fell to 86.1% of the unincubated control values ($P < 0.01$). The release of renin increased from 26.9% (control) to 34.7% in the presence of salbutamol ($P < 0.01$). The total content of renin in the salbutamol containing flasks was 120.8% of the unincubated controls compared with 126.9% in the incubated control flasks. These values are not significantly different.

The results may best be compared graphically as in Figure 2. This shows the similarities between all four drugs in stimulating renin release, and shows the differences in mechanism by which this is achieved.

Discussion

The present investigation clearly indicates that isolated renal cortical cells of the cat respond to adrenaline and noradrenaline added to the incubation medium by an increase in renin production, and in this respect resemble dog renal cortical cells (Michelakis *et al.*, 1969). The dog cells may be somewhat more sensitive to the catecholamines, as in the experiments performed by Michelakis and co-workers the amount of adrenaline or noradrenaline added to the flask gave a maximum concentration of about 0.40 x 10^{-4}M. In the present experiments, concentrations of 1.09 and 1.18 x 10^{-4}M were used, although we have observed effects with concentrations as low as 0.44 x 10^{-4}M.

It should be stressed that these *in vitro* effects of catecholamines are produced with concentrations in the medium about 1000 times the normal plasma levels. If this relative insensitivity to the action of the catecholamines of the isolated renal cortical cells is not a methodological artefact, the implication of these results, in both species, is that circulating catecholamines do not stimulate renin release *in vivo.* It is possible, of course, that such high concentrations are achieved in the region of the nerve terminals following renal nervous activity. Such terminals have been demonstrated in close proximity to the renin-containing juxtaglomerular cells (Barajas & Latta, 1967). However, it is possible that the use of collagenase in the preparation of the cells may have destroyed membrane receptors, or that the artificial medium was not optimal for the functional activity of the cells. We have not found kidney slices, which were not exposed to the collagenase, to be any more sensitive to the catecholamines than the isolated cells (unpublished observations). De Vito, Gordon, Cabrera & Fasciolo (1970) have studied renin release by kidney slices obtained from rats and were unable to demonstrate renin release with concentrations of 0.11 x 10^{-4}M. However, this dose level was found to be effective in causing renin release by Rosset & Veyrat (1971) using slices from human kidneys removed for a variety of pathological conditions.

We have found that, when the cortical cells were incubated for 2 h in the absence of added catecholamine, there was an increase in the total renin content of the flask, and that the entire increase was found in the medium and never in the cells. The mean increase in renin content during incubation of all the controls of the present experiments was 27.2%. Also, when either adrenaline or noradrenaline was added to the medium the increase in total renin content observed after incubation was primarily present in the medium. The addition of noradrenaline resulted in an increase in renin production of 45% compared with incubated controls. With adrenaline the increase was 34%. The effect of the catecholamines was significant.

The effects of isoprenaline and salbutamol on renin production by renal tissue *in vitro* have not been previously reported. In our preliminary studies, in which we measured total renin content of the incubation flasks, we were unable to demonstrate an effect of isoprenaline. However, when cell content and the amount released into the medium were estimated separately it became clear that, although a significant effect on renin production could not be demonstrated, isoprenaline had a potent effect in causing the release of cellular renin. Salbutamol, though less effective than isoprenaline, had a similar effect in stimulating the release of renin without enhancing production. Clearly, the two naturally-occurring substances affect the renin-producing cells in a different way from that of the synthetic compounds. The results are compatible with a membrane effect, by the synthetic compounds, which permits release of stored renin. The naturally-occurring substances may, on the other hand, only be stimulating renin synthesis with release following in a passive manner, depending on concentration gradients. They could also be active in inducing renin release by the same mechanism which mediated the action of the synthetic drugs. Evidence from *in vivo* studies indicates that renin release in response to renal nerve stimulation (Coot *et al.*, 1972; Loeffler *et al.*, 1972) is blocked by propranolol, indicating that the β-adrenoceptor plays an important role in the release of renin initiated by the natural transmitter.

There is little evidence regarding the biochemical events within the juxtaglomerular cells following catecholamine stimulation. Michelakis *et al.* (1969) have suggested that these substances may act by inducing the formation of cyclic AMP, as addition of this compound to the incubation medium resulted in an increase in renin production. However, in order to study the subject directly, the renin-producing cells, which constitute a very small proportion of the cells in the preparation, would have to be separated from the other cells. This could present formidable problems.

In the present study salbutamol was about one third as potent as isoprenaline in stimulating renin release *in vitro*. Comparisons of the effects of isoprenaline and salbutamol on femoral blood flow have indicated salbutamol to be one tenth as potent as isoprenaline (Cullum, Farmer, Jack & Levy, 1969), one eighth as potent (Kofi Ekue, Shanks & Zaidi, 1971) or about one third as potent (Daly, Farmer & Levy, 1971). Salbutamol has been reported to be over 100 times less potent than isoprenaline in increasing heart rate (Daly *et al.*, 1971). Current work in our laboratory, using the cardioselective blocker ICI 66082, suggests that this drug is less potent in inhibiting renin release in response to renal nerve stimulation than propranolol, although it has been reported to be equipotent in blocking the increase in heart rate due to isoprenaline (Barrett, Carter, Fitzgerald, Hull & Le Count, 1973). Although considerably more study of the renin producing and releasing mechanisms is required, these results may indicate that the receptors involved in renin release are more like the peripheral vascular than the cardiac receptors.

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STIMULATION OF THE HYPOTHALAMO–PITUITARY–ADRENAL AXIS BY COMPOUNDS FORMED IN INFLAMED TISSUE

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1 Rat paws were injected with carrageenin, and their subcutaneous tissue perfused 135 min later. These perfusates were injected intravenously into receptor rats in which they caused an attenuation of inflammatory responses.

2 The effect was not observed in adrenalectomized receptor rats nor in receptors with electrolytic lesions in the median eminence of the hypothalamus but persisted in adrenal-demedullated animals.

3 The active perfusates also induced eosinopenia in normal or adrenal-demedullated animals, but not in adrenalectomized rats, and produced an increase in blood corticosterone with a concomitant decrease in the amounts of adrenal ascorbic acid.

4 The active perfusates did not affect the responses of isolated preparations to histamine, bradykinin, prostaglandins and 5-hydroxytryptamine neither did they elicit changes of the arterial blood pressure in receptor animals.

5 The anti-inflammatory activity present in perfusates from inflamed paws seems to be formed slowly at the site of the developing inflammatory reaction, since perfusates collected 30-65 min after the injection of carrageenin were ineffective, as was plasma taken from donor rats at various time intervals after carrageenin injections.

6 It is suggested that the anti-inflammatory factor present in the active perfusates exerts its action by stimulation of the hypothalamo-pituitary-adrenal axis.

Introduction

Increased blood concentrations of corticosteroids after injections of endotoxins have been reported on several occasions (Melby, Egdahl & Spink, 1960; Moberg, 1971). Experimental and clinical observations have demonstrated that adrenal corticosteroids exert a marked anti-inflammatory activity (Cook & MacDonald, 1951; Ashton & Cook, 1952; Wyman, Fulton & Schulman, 1953; Moon & Tershakovec, 1954; Wyman, Fulton, Schulman & Smith, 1954; Germuth, 1956).

In the present paper the possibility was investigated as to whether local inflammatory processes could stimulate the adrenal secretion of corticosteroids which might limit the spread of the inflammatory reaction.

Methods

Male Sprague Dawley (Holtzman) rats weighing between 250-300 g were used. Unless otherwise indicated the animals were anaesthetized throughout the experiments with pentobarbitone sodium (30-40 mg/kg, i.p.). Surgery was performed under ether anaesthesia.

Production of oedema in rat paws

Carrageenin was dissolved in distilled water and 0.1 ml of the solution (0.5 mg/ml) injected into the subplantar area of one of the hind paws; the other was injected with 0.1 ml of 0.9% w/v NaCl solution (saline). The volumes of the paws up to the tibio-tarsal articulation were determined by plethysmography at varying time intervals with a modification of the apparatus described by Winder, Wax & Been (1957). Results are expressed as percentage increase in relation to the initial volume of the paw. Control values (saline-injected paws) were subtracted from values obtained in carrageenin-injected paws.

Double coaxial perfusion of rat paws

Simultaneous perfusions of the subcutaneous space of both hind paws of the rat were carried out according to Garcia Leme, Hamamura & Rocha e Silva (1970). Tyrode solution kept between 5 and 10° C or at 37° C was used as the perfusion fluid, the flow rate was 1 ml/10 minutes. Perfusions lasted for 35 min and were performed at various time intervals after carrageenin or saline injections into the paws. The perfusates were assayed as indicated below. A group of animals was injected intravenously with Evans blue (20 mg/kg) (1% aqueous solution) 5 min before the perfusions and the amount of dye present in the perfusates estimated by spectrophotometry at 600 nm. Control values (dye present in perfusates from saline-injected paws) were subtracted from test values (dye present in perfusates from carrageenin-injected paws).

Tests on perfusates

Perfusates collected from donor rat paws treated with carrageenin, dextran or saline were injected intravenously (penis vein) into receptor animals (0.5 ml/rat). The following parameters were measured in the receptor rats: (i) oedema developing after an injection of carrageenin into a hind paw; (ii) dye leakage into the inflamed paws; (iii) number of blood eosinophils; (iv) serum corticosterone concentrations; (v) ascorbic acid concentrations in the adrenal glands; (vi) effect of perfusates upon arterial blood pressure. The assays were carried out in normal rats, bilaterally adrenalectomized rats, bilaterally adrenal-demedullated rats and rats with electrolytic lesions in the median eminence of the hypothalamus. The perfusates were also tested on the guinea-pig isolated ileum and rat uterus.

Blood eosinophils

Blood collected from the tails of non-anaesthetized receptor rats was diluted with Manners solution and the number of eosinophils counted in a Fuchs–Rosenthal chamber (Darmady & Davenport, 1963). Blood samples were collected before and 2 and 4 h after the injection of perfusates. Results are expressed as the percentage deviation from the initial counts.

Determination of serum corticosterone concentrations

Approximately 4 ml blood was collected from the aorta of anaesthetized rats through an opening in the abdominal wall and after clotting, centrifuged at 2,500 rev/min for 10 minutes. Corticosterone was extracted from 0.5 ml serum and estimated according to Guillemin, Clayton, Lipscomb & Smith (1959). Estimations were made 90 min after the intravenous injections of the perfusates or 60 or 180 min after paw injections of either carrageenin or saline. The same method was used to investigate the possible contamination of perfusates collected from donor rat paws with corticosterone.

Determination of adrenal ascorbic acid

The animals were kept isolated in cages for 24 h before the removal of the adrenal glands. At the time of the experiments they were anaesthetized, decapitated and both adrenal glands removed and weighed. Ascorbic acid was extracted and measured according to Mindlin & Butler (1938), 90 min after the intravenous injections of perfusates.

Bilateral adrenalectomy

The adrenal glands were removed under ether anaesthesia by the dorsal approach. The adrenalectomized rats were supplied with saline and water *ad lib.* and were used 48-56 h post-operatively. Within this period of time development of functioning accessory adrenocortical tissue does not occur.

Adrenal demedullation

The glands were exposed under ether anaesthesia and the medulla was expelled through a small incision in the capsule. The animals were used 48-56 h later.

Electrolytic lesion of the median eminence of the hypothalamus

Animals under ether anaesthesia were held in a stereotaxic apparatus (Johnson Sci. Instr. Co.). Through a hole drilled in the skull a stainless steel wire electrode was introduced into the median eminence, using the coordinates described by De Groot (1959). The electrode was isolated with the exception of its tip. The lesion was made by applying a 3 mA current for 10 seconds. The animals were used 36-40 h later. The effectiveness of the brain lesion was tested by the amount of water ingested in the 24 h period following surgery. Those animals with water intakes in excess of 100 ml in the first 24 h after the operation were used (McCann & Fruit, 1957).

Arterial blood pressure

Blood pressure in the carotid artery was measured with a mercury manometer. The perfusates were injected into a cannulated jugular vein.

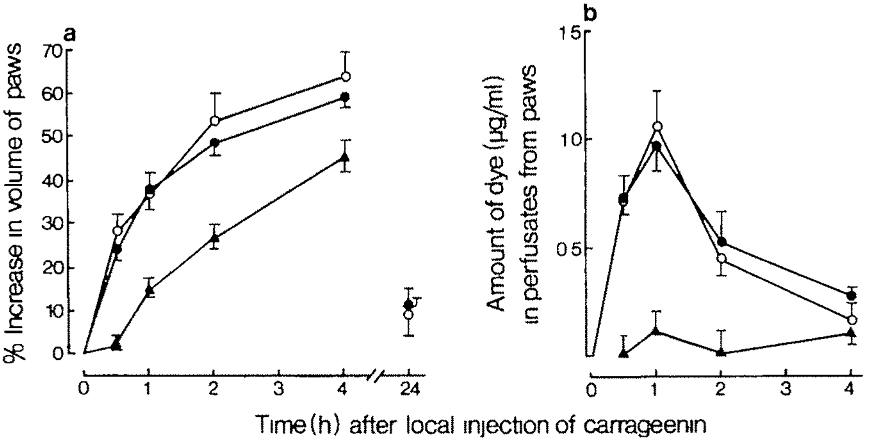

Figure 1 (a) Oedema formation in rat paws after the local injection of 50 µg carrageenin. (b) Dye (Evans blue, injected i.v.) concentration in perfusates from rat paws with carrageenin oedemas. (o) Controls; (▲) rats pretreated with 0.5 ml (i v.) of a perfusate from paws of a donor rat with carrageenin oedema. Perfusate (5-10° C) collected 135-170 min after carrageenin injection. (●) Rats pretreated with perfusate from saline-injected donor rat paws. Each point is mean value; vertical bars show s.e. mean. Oedema experiments started with 26-40 rats in each group. At each time five rats were taken for perfusion experiments and subsequently killed. Perfusates administered to receptor rats immediately before paw injections

Isolated preparations

The guinea-pig ileum was immersed in Tyrode solution in a 10 ml organ bath at 37° C, the rat uterus in de Jalón solution in a 5 ml bath at 28° C.

Drugs

Acetylsalicylic acid (Endosprin, Enila); ascorbic acid (E. Merck AG); Evans blue dye (E. Merck AG); bradykinin (BRS-640, Sandoz); carrageenin, sodium salt, mol wt. 60,000-100,000 (Merck, Sharp & Dohme), corticosterone (Sigma); 5-hydroxytryptamine (Serotonin, Mann Research Lab.); histamine diphosphate (Sigma); indomethacin (Merck, Sharp & Dohme); pentobarbitone sodium (Nembutal, Abbott); prostaglandins E_1 and E_2 (Upjohn); dextran, mol. wt. 60,000-90,000 (Nutritional Biochemical Corp.).

Results

The intravenous administration of 0.5 ml of a perfusate collected from a rat paw injected 135 min previously with carrageenin (active perfusates) attenuated the development of paw oedema induced by an injection of carrageenin in the receptor rats as well as exudation into an inflamed paw of dye previously administered by the intravenous route. Perfusates collected simultaneously from the saline injected control paw of the donor rats were ineffective (Figure 1). In these experiments the perfusion fluid was kept between 5-10° C. If it was kept at 37° C the activity was diminished.

When the perfusates were collected 30 min after the injection of carrageenin into the paw of the donor rat, no oedema reduction was observed in receptor animals (Figure 2). In these experi-

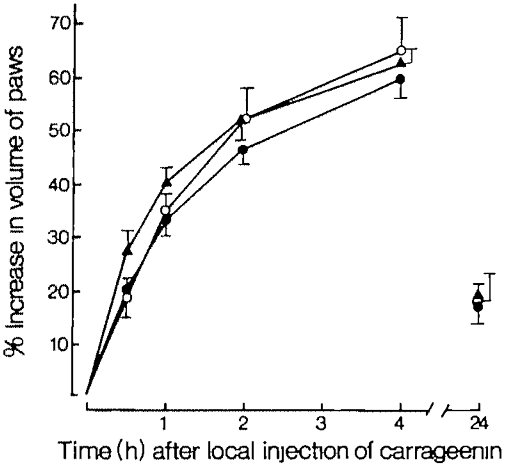

Figure 2 Oedema formation in rat paws after local injection of 50 µg carrageenin. (o) Controls; (▲) rats pretreated with 0.5 ml (i.v.) of a perfusate from paws of a donor rat with carrageenin oedema. Perfusate (5-10° C) collected 30-65 min after carrageenin injection. (●) Rats pretreated with perfusate from saline-injected donor rat paws. Means of 6 controls and 12 pretreated rats in each group. Vertical bars show s.e. mean. Perfusates administered to receptor rats immediately before paw injections.

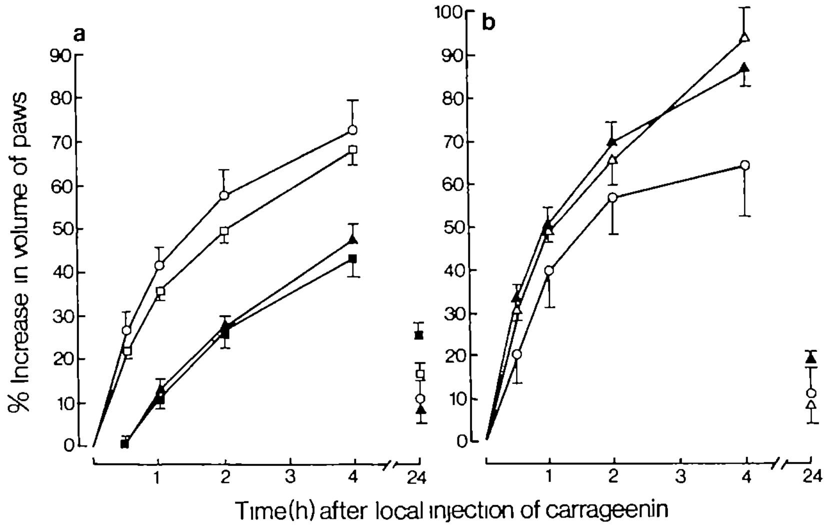

Figure 3 Oedema formation in rat paws injected with 50 μg carrageenin. (a) Results obtained in untreated rats (○); or in rats pretreated with 0.5 ml perfusates collected from donor rat paws injected with 50 μg carrageenin intact (▲), adrenalectomized (□); adrenal-demedullated (■). (b) Results obtained in untreated rats (○) and in rats submitted to a lesion of the median eminence of the hypothalamus receiving (△) and not receiving (▲) 0.5 ml perfusates as in (a). Perfusates (5-10° C) collected 135-170 min after carrageenin injections in the donor animals and administered intravenously to receptor animals immediately before carrageenin. Each point is mean value (n = 7 for ○; 9 for ▲; 15 for □, 10 for ■; 5 for △ and ▲ in (b)) Vertical bars indicate s.e mean.

Table 1 Changes in the number of circulating eosinophils in rats receiving 0.5 mg corticosterone or 0.5 ml perfusates collected from donor rat paws injected with carrageenin (active perfusate) or saline (inactive perfusate).

	Blood eosinophils: % Deviation from the initial mean value	
Treatment	*2 h after injections*	*4 h after injections*
	Normal rats	
Corticosterone	−17.6 ± 5.5	−40.4 ± 3.8 (5)
Active perfusate	−33.7 ± 3.8	−48.3 ± 4.1 (5)
Inactive perfusate	+25.6 ± 8.5	+31.6 ± 5.2 (5)
Controls	+14.8 ± 12.4	+18.4 ± 9.9 (5)
	Adrenalectomized rats	
Corticosterone	−18.6 ± 2.9	−31.5 ± 3.6 (5)
Active perfusate	−0.7 ± 6.1	−0.6 ± 2.9 (5)
Inactive perfusate	+8.0 ± 5.0	+8.5 ± 6.3 (4)
Controls	+39.0 ± 16.4	+43.9 ± 17.8 (5)
	Adrenal-demedullated rats	
Corticosterone	−22.3 ± 4.2	−44.2 ± 3.5 (5)
Active perfusate	−33.8 ± 7.0	−52.4 ± 7.0 (5)
Inactive perfusate	+2.5 ± 4.7	+13.0 ± 7.9 (5)
Controls	+19.7 ± 18.5	+8.7 ± 6.2 (4)

Mean values with s.e. mean are given Figures in parentheses Indicate number of animals in each group Corticosterone was given in oil-suspension (s.c.), perfusates (given i.v) were collected between 135 and 170 min after carrageenin injections into the paw of the donor animals. The initial number of circulating eosinophils/mm^3 was 241 ± 25; 390 ± 62 and 373 ± 44 (mean ± s.e. mean) respectively in normal, adrenalectomized or adrenal-demedullated animals.

ments the perfusion fluid was kept between 5-10° C.

Similar results were observed when dextran (100 μg) was injected as an irritant in the donor rat paw and the perfusates collected were given intravenously to a receptor rat whose paw was injected with carrageenin.

Active perfusates also reduced carrageenin oedema in receptor animals which were bilaterally adrenal-demedullated but showed no effect in adrenalectomized rats or in rats with lesions in the median eminence of the hypothalamus (Figure 3). However, bilateral adrenalectomy and bilateral adrenal-demedullation did not influence the development of oedema when such rats were injected with carrageenin.

The intravenous injections of active perfusates, but not of those collected from saline-injected paws, produced a reduction in the number of circulating eosinophils in either normal receptor rats or in rats submitted to bilateral adrenal-demedullation. This action was not observed in bilaterally adrenalectomized animals. The eosinopenia was more intense than that observed after the subcutaneous injection of 0.5 mg corticosterone in oil suspension (Table 1). The number of eosinophils was counted 2 and 4 h after the injections of either the perfusates or corticosterone.

Table 2 Serum corticosterone concentrations in rats receiving 0.5 ml perfusates collected from donor rat paws injected with carrageenin (active perfusate) or saline (inactive perfusate)

Treatment	*Corticosterone (μg/100 ml serum)*
Active perfusate	20.09 ± 2.22 (11) ($P < 0.01$)*
Inactive perfusate	9.76 ± 2.18 (8) ($P > 0.05$)*
Controls	10.28 ± 1.71 (7)

Estimations were made 90 min after intravenous injection of perfusates. Mean values with s.e. mean are given. Number of animals in each group are shown in parentheses. All animals were anaesthetized with pentobarbitone sodium. Perfusates were collected between 135 and 170 min after paw injections in the donor animals. * Student's *t* test, by comparison with control values.

Increased concentrations of blood corticosterone (Table 2) and a decrease in the concentration of adrenal ascorbic acid (Table 3) were detected 90 min after the intravenous injections of active perfusates into normal rats. Perfusates collected from saline-injected paws of donor rats had no effect.

When instead of perfusates, plasma was collected from donor rats 30, 60 or 120 min after carrageenin and 0.5 ml injected intravenously into receptor animals, no attenuation of the carrageenin oedema was observed up to 4 h after the injection.

The active perfusates produced no arterial blood pressure changes in normal receptor animals; they did not elicit any response of the guinea-pig isolated ileum or rat uterus and did not interfere with the response of these tissues to histamine, bradykinin, prostaglandins E_1 and E_2 (guinea-pig ileum) or 5-hydroxytryptamine (rat uterus). No steroids could be detected in the active perfusates. The method employed easily detects 0.02 μg corticosterone/ml of the acid extract.

Subcutaneous injections of 1 mg corticosterone/rat (in oil suspension) 90 min before plantar administration of carrageenin, or intraperitoneal injection of acetylsalicylic acid (100 mg/kg) or indomethacin (10 mg/kg) 30 min

Table 3 Adrenal ascorbic acid in rats receiving 0.5 ml perfusates collected from donor rat paws injected with carrageenin (active perfusate) or saline (inactive perfusate).

Treatment	*Ascorbic acid (mg/100 g adrenal tissue)*
Active perfusate	282.87 ± 10.56 (7) ($P < 0.01$)*
Inactive perfusate	392.61 ± 28.41 (10) ($P > 0.05$)*
Controls	380.05 ± 10 28 (7)

Estimations were made 90 min after intravenous injections of perfusates. Mean values with s.e. mean are given. Number of animals in each group are shown in parentheses. All animals were anaesthetized with pentobarbitone sodium. Perfusates were collected between 135 and 170 min after paw injections in the donor animals. * Student's *t* test, by comparison with control values.

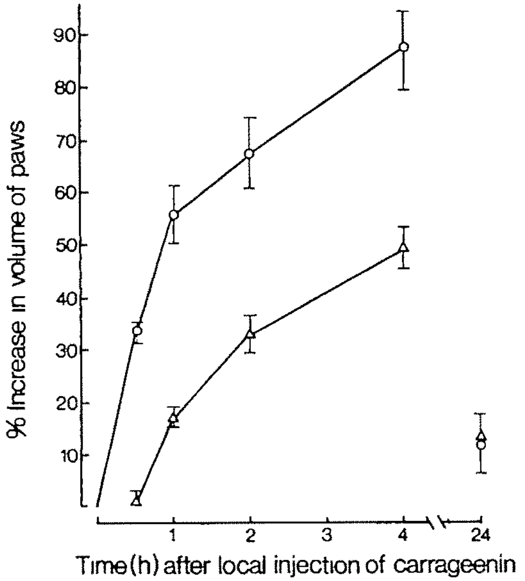

Figure 4 Effect of corticosterone (1 mg in oil, s.c.) on the development of carrageenin-induced rat paw oedema. Carrageenin (60 µg) injected locally 90 min after the steroid. (o) Controls; (△) corticosterone-treated rats. Each point is mean value (n = 6 for o; n = 9 for △). Vertical bars show s.e. mean.

before carrageenin, reduced the resulting paw oedema to a similar extent as the active perfusates (Figures 4 and 5). In contrast to the active perfusates, acetylsalicylic acid and indomethacin were also effective in bilaterally adrenalectomized animals.

When carrageenin was injected into one of the hind paws of normal untreated rats and the other paw was injected with this irritant after a 135 min interval, the oedema caused in the latter was much smaller than that produced in the former. This attenuation of responses was not seen in adrenalectomized rats whose hind paws were injected with carrageenin keeping the 135 min interval between both injections (Figure 6). Furthermore 67% increase in the serum corticosterone concentration was observed in four normal rats 180 min after paw injections of carrageenin (15.0 ± 1.0 µg/100 ml serum; mean with s.e. mean) as compared to three normal rats injected with saline (9.0 ± 0.48 µg/100 ml serum). This increase was not seen 60 min after carrageenin injections.

Discussion

Our results show that perfusates collected from donor rat paws locally injected with carrageenin

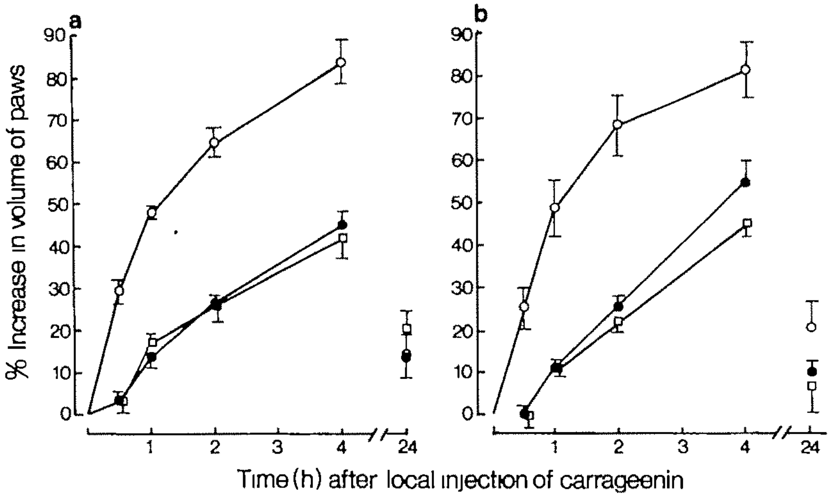

Figure 5 (a) Effect of acetylsalicylic acid (100 mg/kg, i.p.) or (b) indomethacin (10 mg/kg, i.p.) on the development of the carrageenin-induced rat paw oedema. Carrageenin (50 µg) injected locally 30 min after the anti-inflammatory drugs. (o) Untreated controls; (●) intact, drug-injected rats, (□) adrenalectomized, drug-injected rats. Each point is mean value (n = 5). Vertical bars show s.e. mean.

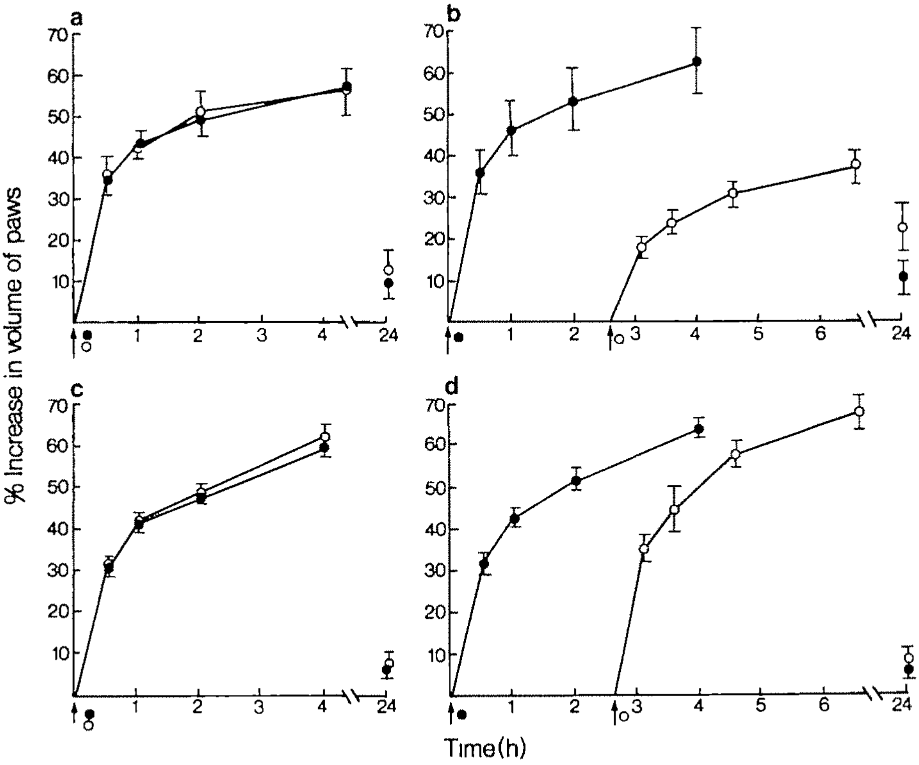

Figure 6 Oedema formation in rat paws after local injection of 50 μg carrageenin. (a) and (b) Results obtained in normal rats. (c) and (d) Results obtained in adrenalectomized rats. In (a) and (c) both hind paws were injected simultaneously with the irritant, in (b) and (d) right paws were injected 135 min after left paws. (○) Right paw; (●) left paw. Arrows indicate carrageenin injections. Each point is mean value (n = 6). Vertical bars show s.e. mean.

135 min previously can attenuate inflammatory responses in receptor rats. This effect was not observed in adrenalectomized receptor rats or in receptors with electrolytic lesions in the median eminence of the hypothalamus. It persisted in animals which had been adrenal-demedullated. The active perfusates also induced eosinopenia in normal or adrenal-demedullated animals, but not in adrenalectomized rats. Furthermore, they increased blood concentrations of corticosterone and produced a decrease in the adrenal ascorbic acid content.

The perfusates did not affect the responses of the isolated tissues to endogenous permeability factors such as histamine, bradykinin, prostaglandins and 5-hydroxytryptamine and did not elicit arterial blood pressure changes in receptor animals.

These results suggest that a factor was present in the active perfusates which could be transferred from donor to receptor animals, where it exerted an anti-inflammatory effect. The active factor probably stimulated the hypothalamo-pituitary-adrenal axis resulting in the release of adrenal corticosteroids (corticosterone). Catecholamines release from the adrenal glands did not play an important role in the phenomenon, as the perfusates were still active in adrenal-demedullated animals.

The formation of the anti-inflammatory substance or substances depended on the existence of a developing inflammatory reaction as shown by the fact that perfusates collected from rat paws injected with saline alone were ineffective. The anti-inflammatory activity detected in perfusates from inflamed paws seems to be formed slowly at the site of the developing inflammatory reaction, as perfusates collected between 30-65 min after the application of the noxious stimulus were not yet effective. Also, plasma taken from donor rats at various time intervals following carrageenin injections into the paws was devoid of anti-inflammatory activity. This suggests that the factor responsible for the observed anti-inflammatory effect is concentrated in the inflamed tissues and is slowly released into the circulation.

It is unlikely that the factor in the perfusate from the carrageenin-injected paw is a metabolite of carrageenin, as perfusates from dextran-injected paws were similarly effective.

The mechanism of the anti-inflammatory effect described is different from that observed with acetylsalicylic acid or indomethacin, as these drugs were also active in adrenalectomized rats.

Furthermore, the injection of carrageenin into one of the hind paws of the rats, led to an attenuation of the response to this irritant injected in the other paw 135 min later. This attenuation was not observed in adrenalectomized rats. Our data therefore suggest the existence of a neuro-endocrine feed-back system during the development of acute inflammatory reactions: through local changes in the affected tissues, probably by cell disruption, protein breakdown, or enzymatic activation, a factor is formed which is released into the circulation and stimulates the hypothalamo-pituitary-adrenal axis resulting in the release of corticosterone. The effectiveness of this steroid in reducing oedema formation after carrageenin injection was observed in the present work. Germuth (1956) has suggested that the vaso-constrictive action of corticosteroids is their most important effect in counteracting inflammation. Moberg (1971) reported that the increase of plasma corticosterone resulting from injection of endotoxins was abolished in rats by electrolytic lesions of the median eminence of the hypothalamus or by a pharmacological blockade of the hypothalamic release of corticotropin-releasing factor. It has also been suggested that the anti-oedema activity of tetrahydrocannabinol was mediated by stimulation of the pituitary-adrenal axis since it was attenuated in adrenalectomized and in hypophysectomized rats (Sofia, Nalepa, Harakal & Vassar, 1973).

Endogenous anti-inflammatory factors have previously been detected in inflammatory exudates or plasma (Rindani, 1956; Di Pasquale & Girerd, 1961; Di Pasquale, Girerd, Beach & Steinetz, 1963; Robinson & Robson, 1964; Bonta & De Vos, 1969; Billingham, Robinson & Robson, 1969; Bonta, Bhargava & De Vos, 1970; Elliot, Ford-Hutchinson, Harford, Insley, Smith & Sturgess, 1973). Most of these anti-inflammatory factors were detected some days after the institution of an inflammatory response. From our present results it seems likely that the organism is also able to produce 'anti-inflammatory' factors at an earlier stage which help to limit the development of the inflammatory focus. These factors are produced by the inflamed tissue itself and stimulate the release of corticosteroids.

Garcia Leme, Hamamura, Leite & Rocha e Silva (1973) observed that after carrageenin injection in the rat's paw a dissociation in time exists between oedema formation and leakage of dye previously given intravenously. while the oedema developed slowly, reaching a maximum after 4-5 h, protein-dye-leakage was already maximal after 1 hour. It is possible that the adrenal corticosteroids are responsible for this dissociation by reducing protein-leakage through a vaso-constrictive action. Schayer (1963, 1964, 1967) assumed that the anti-inflammatory action of glucocorticoids was dependent on the antagonism of dilator effects exerted upon the microcirculatory bed in the affected tissue. In this theory the chief vaso-dilating agent was 'induced histamine'.

It should be remembered that since the work by Hench, Kendall, Slocumb & Polley (1949, 1950) it is known that several clinical and biochemical aspects of rheumatoid diseases are improved by the use of corticosteroids or adrenocorticotrophic hormone. Such agents might be reinforcing a naturally occurring situation.

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THE POSSIBLE OCCURRENCE OF ENDOGENOUS ANTI-INFLAMMATORY SUBSTANCES IN THE BLOOD OF INJURED RATS

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1 Using the carrageenin rat paw oedema test as an assay, an attempt has been made to confirm the presence of anti-inflammatory activity in the blood of rats with a chronic inflammatory lesion induced by a polyester sponge, and to relate such activity to the systemic anti-inflammatory effect exerted *in situ* by the lesion. In addition, plasma from rats given acetic acid intraperitoneally has been examined for anti-inflammatory properties.

2 The activity of serum obtained from sponge-bearing adrenalectomized Wistar rats did not differ significantly from that obtained from animals without implants. Furthermore, implanted sponges exerted no systemic anti-inflammatory effect.

3 Similar experiments were performed in sponge-bearing adrenalectomized Sprague-Dawley rats, plasma instead of serum being examined for anti-inflammatory activity. The plasma removed from such animals possessed no anti-inflammatory activity, whilst implanted sponges exerted a small, yet significant, systemic inhibitory effect.

4 When the irritancy of sponge implants was augmented with croton oil, more marked systemic anti-inflammatory effects were observed. However, the plasma obtained from rats injured in this manner exerted no anti-inflammatory effect.

5 No anti-inflammatory activity could be detected in plasma samples obtained from rats treated with doses of acetic acid capable of producing pronounced systemic anti-inflammatory effects.

6 It was concluded that the induction of these inflammatory lesions in rats does not appear to lead to a detectable release of endogenous anti-inflammatory substances into the circulation.

Introduction

The hypothesis that irritants exert their systemic anti-inflammatory action by releasing into the blood stream a factor or factors capable of inhibiting inflammation at distant sites is supported by observations that the blood of some injured rats possessed anti-inflammatory properties (Laden, Blackwell & Fosdick, 1958. Robinson & Robson, 1964; Billingham, Robinson & Robson, 1969). Furthermore, Goldstein, Schemano, Demeo & Beiler (1967) found in parabiotic rats that the systemic anti-inflammatory activity of intraperitoneally administered kaolin was effected via the blood.

As part of a study designed to elucidate the mechanisms involved in the systemic anti-inflammatory actions of irritants, some of the evidence favouring a humoral mechanism of action has been re-examined. In particular, attempts have been made to confirm the presence of anti-inflammatory activity, detected by Robinson & Robson (1964) and Billingham *et al.* (1969) in the blood of rats bearing a chronic inflammatory lesion caused by the subcutaneous implantation of polyester sponges, and to relate such activity to the systemic anti-inflammatory effects exerted *in situ* by implanted sponges. In addition, the plasma removed from rats acutely injured by acetic acid has been examined for anti-inflammatory properties.

Methods

Animals

In attempts to verify the experiments of Billingham *et al.* (1969) Wistar rats (A.S.L.) were used; in other experiments, Sprague-Dawley rats were employed for both blood sampling and evaluation of anti-inflammatory activity. All animals were males weighing 120-255 g.

Bilateral adrenalectomy was performed through a dorsal midline incision, under ether anaesthesia. The skin wound was closed with Michel suture clips. Operated animals were maintained on Spillers' Laboratory Small Animal (Autoclaved) diet and 0.9% w/v NaCl solution (saline) until they were required for further experimentation on the fourth day after the operation.

In some animals, polyester sponges (50 x 20 x 7 mm) were implanted subcutaneously, one per rat, via a dorsal skin incision (in adrenalectomized rats, this was performed immediately following the operation).

Collection and preparation of plasma and serum

Animals were anaesthetized with ether, the abdominal cavity was opened and blood was withdrawn from the inferior vena cava, using a blood sampler (Peel-A-Way Scientific, South El Monte, California) fitted with a No. O serum needle. For plasma collection, the sampler tube had previously been rinsed with heparin solution (500 u/ml) and the needle deadspace was filled with this solution at the time of sampling. Blood samples were cooled and centrifuged at approximately 800 g for 40 min at 0-5° C and the plasma removed. Overtly haemolysed samples were rejected. Plasmas from rats receiving the same pretreatment were pooled, freeze-dried and stored below 0° C. Serum samples were obtained similarly but the sampler contained no anticoagulant. Following withdrawal, the blood was transferred to a glass centrifuge tube, cooled in ice, and then centrifuged at approximately 800 *g* for 15 min at 0-5° C. Pooled sera were then poured into lengths of dialysis tubing and dialysed against 4 litre batches of 0.9% w/v NaCl solution (saline) for approximately 64 h at 2-4° C (saline batches were changed about every 24 h). The contents of the dialysis sacs were then freeze-dried and stored below 0° C. When required for use, freeze-dried plasma or serum was dissolved in water (Water for Injection, B.P.). Unless otherwise stated, the doses of plasma or serum stated in **Results** refer to the amount of freeze-dried material injected per kg.

Evaluation of anti-inflammatory activity

The anti-inflammatory activity of serum samples obtained from Wistar rats was determined in rats of the same strain using the carrageenin rat paw oedema test (Winter, Risley & Nuss, 1962) in a manner similar to that employed by Billingham *et al.* (1969). The samples were administered s.c. immediately following the subplantar carrageenin injection (0.1 ml of a 1% w/v suspension in 0.9% w/v saline; increase in left hind paw volume measured 5 h later). Control animals received a similar dose volume (10 ml/kg) of 0.9% w/v saline. Food and water were withdrawn immediately before the start of the experiment.

Systemic anti-inflammatory activity in Wistar rats with implanted sponges was determined 4 days after implantation by injecting the left hind paw of each rat with 0.1 ml of a 1% w/v carrageenin suspension. The subsequent increase in paw volume was measured 5 h later. The oedema obtained in sponge-bearing rats was expressed as a percentage of that obtained in appropriate control animals. Food and water were withdrawn immediately before the start of the experiment.

The activity of plasma samples obtained from Sprague-Dawley rats was determined in similar rats using the carrageenin paw oedema method described by Atkinson, Boura & Hicks (1969). The samples were administered either i.p or s.c. 1 h before the subplantar injection of carrageenin (0.05 ml of a 1% w/v suspension); the increase in left hind paw volume was measured 3 h later. Control animals received a similar dose volume (10 ml/kg) of the vehicle.

Systemic anti-inflammatory activity in Sprague-Dawley rats with implanted sponges was determined by injecting into their paws 0.05 ml of a 1% w/v carrageenin suspension and measuring the oedema 3 h later

The statistical significance of differences between test groups was calculated using the two-tailed Student's *t*-test. Results were considered significant if $P \leqslant 0.05$.

Results

Degree of anti-inflammatory activity induced in sponge-implanted adrenalectomized rats

In attempts to verify the experiments of Billingham *et al.* (1969), serum was collected from adrenalectomized Wistar rats bearing implanted sponges on the fourth day after the operation. The anti-inflammatory activity of prepared pooled samples was investigated and compared with that of a similar dose (600 mg/kg) of serum from rats without sponges. The results (Table 1) showed that the sera from rats, both with and without implants, produced a significant anti-inflammatory effect when compared with saline-treated rats. However, the degree of activity produced by each serum sample did not differ significantly.

To ascertain the systemic influence of sponge implantation *in vivo*, inhibition of carrageenin oedema was determined directly in adrenalectomized sponge-bearing rats as described above; adrenalectomized rats without sponges were used as controls. The results (Table 2) showed that the implanted sponges exerted no significant anti-inflammatory effect.

In similar experiments with Sprague-Dawley rats, plasma, but not serum, was collected from donor rats 4 days after sponge implantation. Anti-inflammatory activity was evaluated by the method of Atkinson *et al.* (1969).

Under these conditions neither control nor test plasma samples exerted significant anti-inflammatory activity when doses of 600 and 1000 mg/kg were injected i.p. and s.c., respectively (Table 1). However, sponge implantation exerted *in vivo* a small but significant systemic anti-inflammatory effect (Table 2).

Anti-inflammatory activity exerted by sponge implantation and croton oil in adrenalectomized and in intact Sprague-Dawley rats

As the failure to detect significant anti-inflammatory activity in serum or plasma from sponge-bearing rats was associated with only weak systemic activity, it was possible that the implanted sponges were not producing a

Table 1 Anti-inflammatory effect of serum or plasma obtained from adrenalectomized rats 4 days after the subcutaneous implantation of polyester sponges, as measured against carrageenin-induced rat paw oedema

Treatment	*Dose*	*Mean increase in paw volume ± s.e. mean (ml)*	*% Inhibition of controls*	P	
				vs. controls	*vs. donor control serum/plasma*
Wistar rats*					
0.9% w/v saline (controls)	10 ml/kg, s.c.	0.83 ± 0 05†			
Non-implanted rat serum	600 mg/kg, s.c.	0.70 ± 0.04	17	<0.05	
Implanted rat serum		0.61 ± 0.05	26	<0.01	NS
Sprague-Dawley rats**					
(a) Water for Injection, B.P (controls)	10 ml/kg, i.p.	0.57 ± 0.04			
Non-implanted rat plasma (donor controls)	600 mg/kg, i p	0.62 ± 0 05	–	NS	
Implanted rat plasma		0.58 ± 0.04	–	NS	NS
(b) Water for Injection, B.P. (controls)	10 ml/kg, s.c.	0.54 ± 0.04			
Non-implanted rat plasma (donor controls)	1000 mg/kg, s.c.	0.58 ± 0.05	–	NS	
Implanted rat plasma		0.61 ± 0.05	–	NS	NS

Each result is the mean of 10 observations (†9) N.S. = not significant ($P > 0.05$).

* Paw volume increases measured 5 h after subplantar injection of 0.1 ml of 1% w/v carrageenin suspension.

** Paw volume increases measured 3 h after subplantar injection of 0.05 ml of 1% w/v carrageenin suspension.

Table 2 Systemic anti-inflammatory effect 4 days after subcutaneous implantation of polyester sponges into adrenalectomized rats, as measured against carrageenin-induced paw oedema

Pretreatment	*Mean increase in paw volume ± s.e. mean (ml)*	*% Inhibition of controls*	P *vs. controls*
Wistar rats*			
Adrenalectomy alone (controls)	0.98 ± 0.05		
Adrenalectomy + sponge implanted	0.92 ± 0.05	6	NS
Sprague-Dawley rats**			
Adrenalectomy alone (controls)	0.62 ± 0.03		
Adrenalectomy + sponge implant	0.51 ± 0.03	19	<0.02

Each result is the mean of 10 observations. NS = not significant ($P > 0.05$).

* Paw volume increases measured 5 h after subplantar injection of 0.1 ml of 1% w/v carrageenin suspension.

** Paw volume increases measured 3 h after subplantar injection of 0.05 ml of 1% w/v carrageenin suspension.

sufficiently powerful irritant stimulus. To test this possibility, the sponge-induced inflammation was augmented by the s.c. injection of 0.1 ml of croton oil in close proximity to the sponge immediately following implantation.

On the fourth day after treatment, either plasma samples were obtained or systemic anti-inflammatory effects were measured. The results showed that the combination of sponge implantation and croton oil injection exerted a marked systemic anti-inflammatory effect in adrenalectomized rats (Table 3). However, only 6 out of 20 croton oil-treated rats survived the 4-day post-operative period, and 4 of the survivors were moribund by the end of the experiment. It was therefore possible that reduced oedema could have been due mainly to the poor health of the animals rather than a genuine influence of the irritancy. No attempt was made to harvest plasma from these rats because large numbers of animals would be required to obtain adequate volumes of pooled plasma. The experiments were therefore repeated in intact rather than in adrenalectomized rats; control animals received a dorsal skin incision only. Systemic anti-inflammatory activity was determined 4 days after treatment as before. Table 3 shows that systemic activity was less than that observed in adrenalectomized animals but was apparently greater than that induced by implanted sponges alone. All animals survived the entire experimental period. Plasma obtained from such animals 4 days after treatment exhibited no significant anti-inflammatory activity (Table 3).

Activity of plasma from acetic acid-treated rats

An attempt to detect anti-inflammatory activity in the plasma of rats with acute inflammation was made using a dose of acetic acid previously shown (Atkinson, 1971) to produce a marked systemic inhibition of carrageenin oedema. Plasma samples were obtained from Sprague-Dawley rats 4 h after i.p. injection of either acetic acid (60 mg/kg, as 10 ml/kg of a 0.6% v/v solution) or Water for Injection, B.P. (10 ml/kg). Table 4 shows that there was no significant anti-inflammatory activity in either control or test plasma, even at doses of 1.2 g/kg. The result was confirmed with a much higher dose of acetic acid (200 mg/kg, i.p.) which produces an approximately 85% inhibition of carrageenin oedema (Atkinson, 1971). Plasma was obtained from such animals 4 h after injection and examined within a few hours of collection. No anti-inflammatory activity was detected in either control or test plasma samples (Table 4).

Table 3 Anti-inflammatory effects of subcutaneous polyester sponges and croton oil (0.1 ml, s.c.) in adrenalectomized and intact Sprague-Dawley rats, as measured against carrageenin-induced rat paw oedema

(1) *Systemic activity (assessed 4 days after treatment)*

Pretreatment	*Mean increase in paw volume ± s.e. mean (ml)**	*% Inhibition of controls*	P *vs controls*
(a) Adrenalectomized rats			
Adrenalectomy alone (controls)	0.74 ± 0.02		
Sponge implant + croton oil	0.38 ± 0.06†	49	<0.001
(b) Intact rats			
Dorsal skin incision alone (controls)	0.61 ± 0.04		
Sponge implant + croton oil	0.46 ± 0.03	24	<0.005

(2) *Activity of plasma (collected 4 days after treatment—dose 1200 mg/kg, s.c.)*

Treatment	*Mean increase in paw volume ± s.e. mean (ml)**	*% Inhibition of controls*	P *vs. controls*	P *vs. donor control plasma*
Water for Injection, B.P., 10 ml/kg, s.c. (controls)	0.65 ± 0.04			
Plasma from intact rats with dorsal skin incisions (donor controls)	0.59 ± 0.03	11	NS	
Plasma from intact rats with sponge implant + croton oil	0.60 ± 0.03	8	NS	NS

Each result is the mean of 10 observations (†6). NS = not significant ($P > 0.05$).

* Measured 3 h after subplantar injection of 0.05 ml of a 1% w/v carrageenin suspension.

Table 4 Anti-inflammatory effect of plasma obtained from Sprague-Dawley rats 4 h after treatment with acetic acid, as measured against carrageenin-induced rat paw oedema

				P	
Treatment	*Dose (i.p.)*	*Mean increase in paw volume ± s.e. mean (ml)**	*% Inhibition of controls*	*vs. controls*	*vs. normal donor plasma*
(1) Freeze-dried plasma from rats given acetic acid, 60 mg/kg, i.p.					
(a) Water for Injection, B.P. (controls)	10 ml/kg	0.54 ± 0.06			
Normal donor plasma	600 mg/kg	0.44 ± 0.03	19	NS	
Acetic acid donor plasma		0.49 ± 0.04	10	NS	NS
(b) Water for Injection, B.P. (controls)	10 ml/kg	0.53 ± 0.04†			
Normal donor plasma	1200 mg/kg	0.47 ± 0.04†	12	NS	
Acetic acid donor plasma		0.45 ± 0.02†	16	NS	NS
(2) Fresh plasma from rats given acetic acid, 200 mg/kg, i.p.					
Water for Injection, B.P. (controls)	10 ml/kg	0.46 ± 0.03			
Normal donor plasma		0.41 ± 0.03	12	NS	
Acetic acid donor plasma		0.54 ± 0.04	—	NS	—

Each result is the mean of 5 observations (†10). NS = not significant ($P > 0.05$).
* Measured 3 h after subplantar injection of 0.05 ml of a 1% w/v carrageenin suspension.

Discussion

In the present investigation, it was not possible, under a variety of conditions, to confirm the occurrence of anti-inflammatory activity in the blood of injured rats. Robinson & Robson (1964) showed that the plasma obtained from adrenalectomized rats with implanted sponges exerted anti-inflammatory activity, measured against cotton pellet granuloma and that an implanted sponge reduced the amount of granulation tissue deposited in the same animal. Billingham *et al.* (1969) also demonstrated marked anti-inflammatory activity in the serum from sponge-bearing rats when this was assayed on carrageenin oedema. However, these authors did not ascertain whether this activity was associated with a systemic effect against carrageenin oedema in similarly-treated animals. In the present investigation, the activity of serum from sponge-bearing rats, obtained from the same source as the animals used by Billingham *et al.* (1969), did not differ significantly from that obtained from animals without implants. Moreover, implanted sponges evoked no demonstrable systemic anti-inflammatory activity in this strain of rat. These results suggest that there was no detectable release into the circulation of an endogenous anti-inflammatory factor and raise the possibility that the serum activity observed by Billingham *et al.* (1969) may have been due to an artefact. There was also no significant anti-inflammatory activity detected in the plasma of sponge-bearing Sprague-Dawley rats although a small, but significant systemic effect was demonstrated in such animals.

These results are consistent with our earlier contention that sponge implantation does not lead to the accumulation of endogenous anti-inflammatory factors in the local sponge exudate (Atkinson *et al.*, 1969; Atkinson & Hicks, 1971; Atkinson, Whittle & Hicks, 1971), as suggested by Billingham *et al.* (1969).

An alternative explanation for the lack of systemic and hence blood-borne activity observed in these experiments is that the particular sponges used were not sufficiently irritant to generate the anti-inflammatory factor when implanted in either Wistar or Sprague-Dawley rats. However, the sponges were essentially the same as those used by Billingham *et al.* (1969). When the sponge-induced inflammation in adrenalectomized Sprague-Dawley rats was augmented by injecting croton oil, the systemic anti-inflammatory activity was considerably boosted. This applied to a lesser extent also to intact rats with sponge and croton oil-induced inflammation. However, the plasma removed from such rats possessed little or no anti-inflammatory activity. DiPasquale, Girerd, Beach & Steinetz (1963) also showed that serum obtained from rats with croton oil-induced granuloma pouches did not exert significant anti-inflammatory effects. It would thus appear that the systemic influence of the severe inflammation used in the present experiments was not associated with a humoral mediator capable of suppressing carrageenin oedema.

In contrast to the previous experiments, very pronounced systemic anti-inflammatory effects can be induced acutely in rats treated with acetic acid (Atkinson, 1971). Yet, no anti-inflammatory activity could be detected in the plasma of these rats. In contrast, Laden *et al.* (1958) reported that blood taken from rats treated with another corrosive irritant, silver nitrate, possessed anti-inflammatory properties, but no supporting data have since been published.

The conflict between the present results and those of Billingham *et al.* (1969) may be possibly resolved by a consideration of the control experiments performed in the respective investigations. In the present study, the control sera and plasma were obtained from adrenalectomized animals, whereas in that of Billingham *et al.* (1969), sera from normal unoperated rats were used. It could be argued that adrenalectomy and its attendant trauma initiates some systemic anti-inflammatory effect, perhaps mediated via a humoral factor, thus masking or at least reducing any possible difference between control and test sera or plasma. In contrast, it would be easier to detect a significant difference between sera obtained from normal rats and those from adrenalectomized sponge-implanted rats. However, adrenalectomy tends to potentiate rather than inhibit carrageenin oedema, at least when assessed against sham-operated controls (Atkinson, 1972), which renders the above explanation unlikely.

Even the more severe inflammatory lesions used in the present study did not appear to lead to the release of endogenous anti-inflammatory substances into the circulation. If the marked serum activity observed by Billingham *et al.* (1969) was due to a humoral substance, then the present results suggest that it does not occur consistently and hence may be of little physiological significance. It is more likely that counter-irritant effects generally depend upon competition between inflammatory lesions for mediators or their precursors, a possibility which is supported by numerous studies (Garcia Leme, Schapoval & Rocha e Silva, 1967; Willoughby, Coote & Turk, 1969; Giroud & Timsit, 1970; Atkinson, 1971; Briseid, Arntzen & Dyrud, 1971).

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THE PREVENTION BY SULPHYDRYL COMPOUNDS OF THE TOXICITY IN THE CAT OF 2,6-DIMETHOXYPHENOL AND ITS MORPHOLINOPROPIONYL ESTER

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1 Intravenous (−)-2,6-dimethoxyphenyl-2-morpholinopropionate hydrochloride (M&B 16,573) produced anaesthesia of short duration in the mouse, rat, rabbit, cat, dog and monkey. In the cat but not in other species, a severe and usually fatal toxic reaction was seen 1-2 h after administration.

2 This toxic reaction but not the anaesthetic properties of M&B 16,573 was prevented by the intravenous administration of cysteine or N-acetylcysteine. Cysteamine or dimercaprol were ineffective.

3 Intravenous administration of 2,6-dimethoxyphenol or 2,6-dimethoxyquinol in the cat produced a response similar to the delayed toxic effects of M&B 16,573 but not preceded by anaesthesia. The toxic effects of these compounds were prevented by cysteine.

4 Intravenous 4-allyl-2,6-dimethoxyphenyl-2-morpholinopropionate hydrochloride produced anaesthesia in the cat without the delayed toxic effects seen after M&B 16,573.

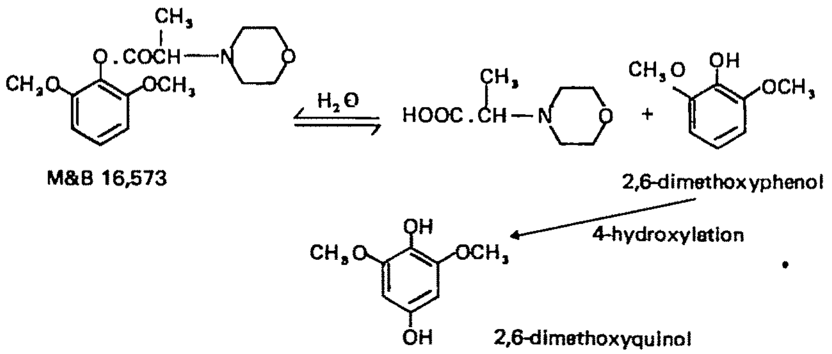
5 The acute toxicity of 2,6-dimethoxyquinol in mice was reduced by the administration of cysteine or N-acetylcysteine.

6 It is postulated that the delayed effects produced by M&B 16,573 in the cat are due to the formation of 2,6-dimethoxyquinol and 2,6-dimethoxybenzoquinone in this species, the toxicity of the latter being reduced by sulphydryl compounds.

Introduction

While evaluating the intravenous anaesthetic properties of a series of basic phenolic esters, and particularly (−)-2,6-dimethoxyphenyl-2-morpholinopropionate hydrochloride (M&B 16,573), it was observed that in the cat but not in the mouse, rat, rabbit, dog or monkey, a delayed toxic and usually fatal reaction was seen. This occurred only after the slow intravenous infusion of approximately twice the anaesthetic dose. The cats appeared to recover normally from the anaesthetic but 0.5-2 h later were seen to be suffering from excessive salivation, facial oedema, swelling of the tongue and respiratory difficulty. The majority of these cats died 2-4 h after administration of the drug.

It seemed likely that M&B 16,573 might be hydrolysed *in vivo* to 2,6-dimethoxyphenol and morpholinopropionic acid and we hypothesized that the phenol might subsequently undergo



4-hydroxylation to form a quinol and the corresponding 2,6-dimethoxybenzoquinone, and that the latter might be a toxic metabolite.

Phillips & Cater (1956) showed that sulphydryl-containing compounds protected against the acute toxicity of menadiol diphosphate (2-methyl-1 : 4-naphthohydroquinone diphosphate) in rats. Hence, if the above hypothesis of the cause for the delayed toxicity of M&B 16,573 were correct, sulphydryl compounds might afford protection against this. Furthermore, this hypothesis suggested that compounds related to M&B 16,573 but suitably substituted in the 4-position of the phenolic ring so that the quinol cannot be formed *in vivo*, should not produce the delayed toxicity seen with M&B 16,573.

This paper describes experiments carried out to test this hypothesis.

Methods

Cats

Cats of either sex weighing 1-2 kg were used. All drugs were administered into a cephalic vein. The cats were kept for 48 h after injection and observed for oedema of face and tongue, respiratory difficulty and colour of urine. The number which died was noted. Cats receiving 0.9% w/v NaCl solution (saline) or other vehicle were used as controls and were studied simultaneously with the cats treated with active drugs.

Mice

Male albino mice (M&B strain) weighing 18-25 g were used. To determine the protective activity of the sulphydryl compounds, mice were injected intravenously with the compound, followed immediately by an intravenous injection of either 2,6-dimethoxyphenol or menadiol sodium diphosphate. The mice dying within 24 h were counted. Control mice receiving only the toxic compound were used in all experiments. The doses of 2,6-dimethoxyphenol or of menadiol sodium diphosphate used produced approximately 80% mortality in control animals.

Experiments in other species

An anaesthetic dose of M&B 16,573 was injected intravenously in the mouse, rat, rabbit, dog and Rhesus monkey and this initial injection was followed by an intravenous infusion to maintain anaesthesia. Animals were observed for 48 h after recovery.

Drugs

The following drugs were used: (−)-2,6-dimethoxyphenyl-2-morpholinopropionate hydrochloride (M&B 16,573); 4-allyl-2,6-dimethoxyphenyl-2-morpholinopropionate hydrochloride (M&B 15,920); 2,6-dimethoxyphenol (Aldrich); 2,6-dimethoxyquinol; Dimercaprol (BAL) injection (Boots); cysteine hydrochloride; cysteamine (2-mercapto-ethylamine hydrochloride); N-acetylcysteine (Airbron, BDH); menadiol sodium diphosphate (Synkavit, Roche); atropine sulphate; promethazine hydrochloride; methysergide hydrogen maleate, dimethothiazine mesylate.

Doses throughout the text refer to the compounds as listed above.

Results

Cat

The rapid intravenous administration of M&B 16,573 100 mg/kg to the cat was followed by general anaesthesia (loss of righting reflex, withdrawal reflexes and corneal reflex). This anaesthesia lasted 2-5 min and was followed by complete and apparently uneventful recovery. No delayed toxic effects were observed at this dose in any of the 12 cats studied. When the initial intravenous injection of 100 mg/kg was followed by an infusion of a further 100 mg/kg over a period of 2-5 min, anaesthesia was maintained throughout the period of drug infusion and for some 5 min afterwards and was followed by apparently complete recovery from anaesthesia. However, after a delay of 0.5-2 h, a characteristic syndrome developed in all these animals. This consisted of facial oedema, swelling of the tongue, respiratory difficulty and, eventually, prostration and death. Urine collected from animals which had received 200 mg/kg M&B 16,573 was dark brown or black in colour. M&B 16,573 (200 mg/kg) was administered in this way to a total of 50 cats. All developed these toxic effects and 37 died within 2-4 h after drug administration.

The precise cause of death was somewhat obscure and, although it involved respiratory difficulty, it was not prevented by intubation and artificial respiration. Post-mortem examination revealed no gross pathological change, other than oedema of the face and tongue. There was no observable oedema of the lung.

Attempts were made to prevent the development of these toxic effects. Atropine (2 mg/kg, 3 cats), promethazine (10 mg/kg, 3 cats), methysergide (1 mg/kg, 2 cats) or dimethothiazine (4 mg/kg, 4 cats) given immediately before M&B 16,573 (200 mg/kg) did not modify the development of the fatal toxic effect.

However, the intravenous administration of cysteine, 200 mg/kg (6 cats) or 600 mg/kg (8 cats) immediately after the infusion of M&B 16,573 (200 mg/kg) completely protected all the animals treated (Table 1). The duration of the anaesthetic activity of M&B 16,573 was unaltered.

Cysteine (600 mg/kg) was also effective when given 1 h (4 cats) or 2.5 h (4 cats) after M&B 16,573, except in one animal in which the signs were already severe when the cysteine was administered 2.5 h after the anaesthetic (Table 1). Other doses of cysteine were not studied.

N-acetylcysteine (200 mg/kg) administered immediately after M&B 16,573 prevented the lethal effects of M&B 16,573 in five of six treated cats (Table 2), whilst cysteamine (300 mg/kg) and dimercaprol (40 mg/kg) were ineffective at the doses used. Cats injected with morpholinopropionic acid in amounts (200 mg/kg) greater than would be formed from M&B 16,573 showed no toxic reaction.

If the toxic effects of M&B 16,573 were due to the formation of 2,6-dimethoxyquinol and 2,6-dimethoxybenzoquinone, similar toxic effects might follow administration of either 2,6-dimethoxyphenol or 2,6-dimethoxyquinol. The intravenous administration of 2,6-dimethoxyphenol (100 mg/kg) to four cats (Table 3) produced no significant anaesthesia but all developed the toxic effects described above and died 1-2 h after the injection. Cysteine (600 mg/kg) given intravenously immediately after 2,6-dimethoxyphenol protected three of the four treated animals (Table 3).

2,6-Dimethoxyquinol (30 mg/kg, 4 cats) produced less severe toxic effects than either M&B 16,573 (200 mg/kg) or dimethoxyphenol (100 mg/kg). However, all the treated animals died and the delay between dosing and death was 20-30 min, as compared with the 30-120 min after M&B 16,573 (Table 3). The toxicity of 2,6-dimethoxyquinol was reduced by cysteine (600 mg/kg). The effects of 2,6-dimethoxybenzoquinone were not studied in the cat because of its poor water solubility.

The intravenous administration of 4-allyl-2,6-dimethoxy-2-morpholinopropionate (M&B 15,920, 200 mg/kg) to three cats produced some

Table 1 Effect of cysteine on the delayed toxicity of M&B 16,573 (200 mg/kg) in the cat

Cysteine		*Number dying/number dosed*		
Dose (mg/kg)	*Time after M&B 16,573 (h)*	*M&B 16,573 + cysteine*	*M&B 16,573 only (control)*	P*
200	0	0/6	4/6	<0.02
600	0	0/8	8/8	<0.001
600	1	0/4	2/3	0.05
600	2.5	1/4	4/4	<0.05

All cats received M&B 16,573 (200 mg/kg intravenously). Control cats received intravenous saline. Cysteine-treated cats received the stated dose of cysteine at the stated time after M&B 16,573.

* Determined by χ^2 test.

Table 2 Effect of sulphydryl compounds on the delayed toxicity of M&B 16,573 (200 mg/kg) in the cat

		Number dying/number dosed		
Sulphydryl compound	*Dose (mg/kg, i.v.)*	*M&B 16,573 + sulphydryl compound*	*M&B 16,573 only (control)*	P*
Cysteine	200	0/6	4/6	<0.02
N-acetylcysteine	200	1/6	4/4	0.01
Cysteamine	300	4/6	5/6	0.5
Dimercaprol	40	6/6	6/8	<0.2

The sulphydryl compounds were administered immediately after M&B 16,573. Data on cysteine are from Table 1 and are included for comparison.

* χ^2 test.

anaesthesia of short duration but none of the animals developed the delayed effects seen after M&B 16,573. The quality of the anaesthesia produced by M&B 15,920 was not so good as that produced by M&B 16,573.

Mouse

Experiments with mice were carried out to obtain more information on the protective action of various sulphydryl compounds.

Intravenous injection of M&B 16,573 (120 mg/kg) was followed by anaesthesia of 1-2 min duration, followed by rapid and uneventful recovery. When the initial injection of 120 mg/kg was followed by a slow intravenous infusion of up to 1000 mg/kg, the duration of anaesthesia was prolonged but the animals recovered with no delayed toxic effects.

In contrast, the LD_{50} of 2,6-dimethoxyquinol for mice was approximately 35 mg/kg. The protective action of various sulphydryl compounds

Table 3 Effect of cysteine (600 mg/kg) on the delayed toxicity of 2,6-dimethoxyphenol and 2,6-dimethoxyquinol in the cat

Drug	*Dose (mg/kg)*	*Number dead/number dosed* *Drug + cysteine*	*Drug alone*	P*
M&B 16,573	200	0/8	8/8	<0.001
2,6-dimethoxyphenol	100	1/4	4/4	<0.05
2,6-dimethoxyquinol	30	1/4	4/4	<0.05

Control cats received the phenol or quinol alone. Treated cats received the phenol or quinol + cysteine (600 mg/kg, i.v.) immediately. Data on M&B 16,573 are from Table 1 and are included for comparison.
* χ^2 test.

Table 4 Protective action of sulphydryl compounds in mice treated with 2,6-dimethoxyquinol or menadiol sodium diphosphate

Sulphydryl compound	*Dose (mg/kg)*	*Mortality as % of mortality in controls* *2,6-dimethoxyquinol*	*Menadiol*
Cysteine	25	75	86
	50	59	72
	100	46	33
	200	0	38
	400	0	26
N-acetylcysteine	31	73	82
	62	60	82
	125	0	65
	250	0	85
	500	0	76
	1000	6	36
Cysteamine	12.5	69	79
	25	72	62
	50	47	24
	100	12.5	0
	200	28	7
Dimercaprol	5	84	100
	10	78	84
	20	125	28
	40	168	14

The sulphydryl compounds were administered immediately before 2,6-dimethoxyquinol (50 mg/kg) or menadiol sodium diphosphate (270 mg/kg). All drugs were administered intravenously. Data obtained from groups of a minimum of 10 mice.

against the acute toxicity of 2,6-dimethoxyquinol was investigated and compared with their ability to reduce the toxicity of menadiol sodium diphosphate. Mice were pre-treated with graded doses of the sulphydryl compound and, immediately afterwards, 2,6-dimethoxyquinol (50 mg/kg) or menadiol sodium diphosphate (270 mg/kg) was administered intravenously. These doses of the quinols killed approximately 80% of un-premedicated mice.

Cysteine and N-acetylcysteine were the most effective of the agents tested in protecting mice from the lethal effects of 2,6-dimethoxyquinol (Table 4). Somewhat surprisingly, however, these compounds did not afford any great degree of protection from the acute toxicity of menadiol sodium diphosphate. In contrast; cysteamine and dimercaprol appeared to be more effective in protecting from the lethal effects of menadiol sodium diphosphate than of 2,6-dimethoxyquinol.

Other species

The anaesthetic properties of M&B 16,573 were also studied in the rat, rabbit, dog and monkey. In these species, anaesthesia of short duration was produced by a suitable intravenous dose of the compound (about 100 mg/kg). This anaesthesia could be prolonged by following the induction dose with a slow intravenous infusion. The total dose infused to each species (number of animals) was: rat (20), 1000 mg/kg; rabbit (4), 400 mg/kg; dog (4), 400 mg/kg; and Rhesus monkey (4), 400 mg/kg. All the animals recovered uneventfully from the anaesthesia and no delayed toxic effects appeared during the following 48 hours.

Discussion

The observation that the lethal reaction described occurred only in cats suggests that, in this species, M&B 16,573 may follow a metabolic pathway which differs from that in the other laboratory animals. M&B 16,573 ((−)-2,6-dimethoxy-2-morpholinopropionate hydrochloride) was designed as a short-acting intravenous anaesthetic whose duration of action was limited by hydrolysis of the ester linkage (Bamford, Biggs, Lee, Owen, Pulsford & Wragg, 1969). The duration of the anaesthetic action of M&B 16,573 in the cat was not different from that observed in the mouse, rabbit, dog or monkey: the hydrolysis of the ester which determines its duration of action is, thus probably common to all the species studied.

The equivalent amount of 2,6-dimethoxyphenol given to cats produced toxic effects identical with those due to M&B 16,573. It seems likely, therefore, that it is in the metabolism of this phenol that cats differ from other species. Robinson & Williams (1958) state that the normal metabolic pathway for phenols involves conjugation with glucuronic acid and that cats do not readily form glucuronides. Dutton & Greig (1957) showed that cat liver slices failed to synthesize glucuronides, although this synthesis was observed in liver preparations from guinea-pig, rabbit, mouse and rat.

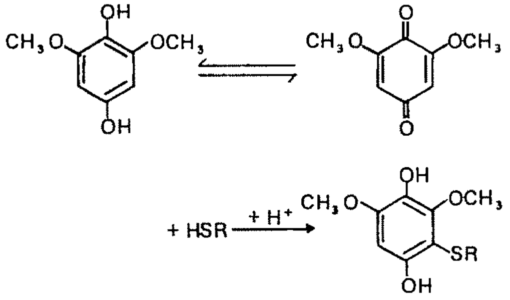
Miller, Powell, Olavesen & Curtis (1973) studied the metabolism of 2,6-dimethoxy $[U\text{-}^{14}C]$phenol in the cat. Following the intravenous administration of 20 mg/kg, 93% of the radioactivity in the urine was identified as 2,6-dimethoxyquinol disulphate, with small amounts of the parent phenol (5%) and a glucuronic acid conjugate (2%). After intravenous administration of 40 mg/kg, similar results were obtained, except that some 3% of the radioactivity in the urine was identified as 2,6-dimethoxyquinol. While sulphate conjugates are the major metabolites from the administration of phenols to cats, this detoxication process may become saturated when higher doses of phenols are administered and the urine might contain more parent phenols, together with their oxidation products (quinones).

In the rat (Miller, Powell, Olavesen & Curtis, 1974), intravenous 2,6-dimethoxy $[U\text{-}^{14}C]$phenol was excreted both as the glucuronide and the sulphate conjugate. The corresponding quinol was not apparently detected.

In the experiments described here, M&B 16,573, 200 mg/kg, given intravenously invariably produced delayed toxic effects. Assuming that most of the dose is hydrolysed, this would be equivalent to an intravenous dose of 94 mg/kg 2,6-dimethoxyphenol. According to the data of Miller *et al.* (1973), some of this 2,6-dimethoxyphenol would be converted to 2,6-dimethoxyquinol. Some of this 2,6-dimethoxyquinol would be detoxified by the formation of sulphate conjugates, but this detoxication process may become saturated, leading to the presence of free 2,6-dimethoxyquinol and possibly the corresponding 2,6-dimethoxybenzoquinone in the circulation.

It is well-known that quinones with unsubstituted positions in the nucleus form addition compounds with substances containing thiol groups (Redfearn, 1965). This reaction accounts for the inhibitory action of certain quinones on the mitochondrial electron transport system (Redfearn & Whittaker, 1962; Smith &

Lester, 1971). Thus, not only would this reaction, e.g. explain the toxicity of 2,6-dimethoxyquinol in the cat, but it would also account for the protective action of sulphydryl compounds.



That the toxicity of M&B 16,573 in the cat was due to the formation of the 2,6-dimethoxyquinol rather than the toxicity of the free phenol was confirmed by the finding that a related compound in which the 4-position was blocked (4-allyl-2,6-dimethoxyphenyl-2-morpholinopropionate) did not produce the delayed toxic effects. Furthermore, Oettel (1936) described a hydroquinone syndrome after the chronic administration of hydroquinone to cats, which is similar to that following M&B 16,573.

The sulphydryl compounds may react with the 2,6-dimethoxybenzoquinone and prevent its interaction with thiol-containing substances involved in mitochondrial electron transport. The differences between the protective action of the sulphydryl compounds examined against the toxicity of 2,6-dimethoxyquinol and menadiol sodium diphosphate may be related to the ease with which these sulphydryl compounds react with the two quinones in question.

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STUDIES ON THE KINETICS OF [^{3}H]-OUABAIN UPTAKE AND EXCHANGE IN THE ISOLATED PAPILLARY MUSCLE OF THE GUINEA-PIG

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1 The uptake, wash-out and exchange of [^{3}H]-ouabain was studied in isolated, resting, and electrically stimulated papillary muscles of the guinea-pig.

2 At the equilibrium level of uptake, a different tissue/medium ratio was obtained for each of the concentrations used, i.e. 3.4, 1.8 and 0.82 for 1×10^{-7} M, 7.7×10^{-7} M, and 5×10^{-6} M ouabain, respectively. Equilibrium was reached more rapidly at high concentrations of ouabain.

3 The maximum number of binding sites for ouabain was estimated to be 1×10^{15} binding sites/g wet weight.

4 No difference in [^{3}H]-ouabain uptake could be detected between resting and electrically stimulated papillary muscles.

5 The kinetics of the ouabain uptake, wash-out and exchange are discussed. The results suggest that there is a saturable compartment in papillary muscle which can best be demonstrated if low concentrations of ouabain are used. Because of its small size, the saturable compartment submerges in the process of the filling of the extracellular space at high concentrations (5×10^{-6} M).

Introduction

In spite of numerous investigations on the positive inotropic action of cardiac glycosides during recent years, their mode of action remains obscure (for review see Lee & Klaus, 1971). In particular, the site of action has been a matter of controversy.

Cardiac glycosides are taken up from the perfusion medium by isolated heart muscle (Kuschinsky, Lahrtz, Lüllmann & Van Zwieten, 1967a; Kuschinsky, Lüllmann, Schmitz & Van Zwieten, 1967b; Kuschinsky, Lüllmann & Van Zwieten, 1968; Dutta, Goswami, Datta, Lindower & Marks, 1968; Dutta & Marks, 1969; Godfraind & Lesne, 1972). However, according to their physico-chemical properties, cardiac glycosides show quite different distribution kinetics. This increases the difficulty of finding a site of binding, common to all digitalis compounds, which could mediate the effects on cardiac muscle.

Dutta *et al.* (1968) and Dutta & Marks (1969) suggested an intracellular site of action, because they found a good correlation between the uptake of digitalis glycosides into a microsomal fraction and their positive inotropic effect. Other authors (Kuschinsky *et al.*, 1967a; 1967b; 1968; Kuschinsky & Van Zwieten, 1969) came to the conclusion that only a modest amount of drug bound 'specifically' by the tissue is essential for its effect and that most of the digitalis glycoside taken up by the tissue is of no pharmacological importance.

Using autoradiographic techniques (Smith & Fozzard, 1963; Conrad & Baxter, 1964; Tubbs, Crevasse & Wheat, 1964; Fozzard & Smith, 1965; Waser, 1965; Löhr, Makoski, Göbbeler & Strötges, 1971) as well as differential centrifugation (Harvey & Pieper, 1955; Spratt & Okita, 1958; Gerber, Fricke, Klaus & Wollert, 1968; Fricke, Gerber, Klaus & Wollert, 1969), much effort was devoted to the intracellular localization of cardiac glycosides and the possible relation to some subcellular structure. However, the results obtained by these methods must be regarded with some caution, because disadvantages of the techniques such as loss of drug from the tissue and redistribution within the cell during the preparation procedures cannot be avoided.

The purpose of the present paper was to gain further evidence for the possible site of cardiac glycoside action. We therefore investigated the uptake of ouabain in order to avoid kinetics which are complicated by a large amount of 'unspecific' accumulation. Papillary muscles were preferred to atria, because they are small ventricular muscle preparations of good integrity. It should also be kept in mind that the morphology of the ventricular muscle is rather different from atrial muscle: it possesses an abundant tubular system continuous with the extracellular space (Sperelakis & Rubio, 1971).

Methods

Right ventricular papillary muscles of less than 1 mm in diameter were dissected from the hearts of guinea-pigs weighing 300-400 grams.

For the determination of the uptake, wash-out and exchange of tritiated compounds, the papillary muscles were suspended vertically in a large organ bath containing oxygenated Tyrode solution at 32° C. A small weight of 340 mg tied to the free end of the muscles adjusted their resting tension. In some experiments the preparations were stimulated by 5 ms square wave impulses of suprathreshold voltage at a rate of 1 Hz. All other experiments were carried out with quiescent preparations.

After an equilibration period of 30 min in Tyrode solution, the muscles were incubated for various periods up to 3 h in a medium which contained the tritiated compound to be investigated. At the end of the incubation period the muscles were assayed for their content of radioactivity. When high concentrations of glycoside were necessary, additional inactive drug was used from a separate stock solution.

In the wash-out experiments, the papillary muscles were preloaded in an incubation medium containing radioactive ouabain and were then washed for various periods in glycoside-free Tyrode solution. In two other experimental groups, the preparations were preloaded for 3 h with either active or inactive ouabain and were then allowed to exchange with identical concentrations of inactive or active ouabain.

The tritium content of the papillary muscles was assayed in the following manner. After well-defined periods of incubation the muscles were removed from the bath, pressed carefully for 90 s between blotting paper and weighed immediately. They were dissolved in 1 ml of Soluene or Hyamine [*p*-(diisobutylcresoxyethyl)-dimethylbenzylammonium hydroxide, 1 M in methanol] at 50° C for 2-4 hours. After addition of 2 ml ethanol and 10 ml scintillation phosphor (4 g 2,5-diphenyl-oxazole (PPO) and 0.5 g 1,4-di-(2(5-phenyloxazole))-benzene (POPOP) dissolved in 1 litre toluene) the samples were counted in a Packard Tricarb Liquid Scintillation Spectrometer. The individual quenching of each sample was corrected by means of an internal tritium standard. The radioactivity of the papillary muscles was expressed as nCi/g tissue (wet weight). The tissue/medium ratio was determined by dividing the radioactivity of the tissue by the radioactivity in the incubation medium measured in nCi/ml. The uptake, wash-out and exchange were plotted as a function of time.

[^{3}H]-ouabain, [^{3}H]-digitoxin and [^{3}H]-sucrose were labelled according to the Wilzbach exchange procedure by New England Nuclear Corporation, Chicago. Purity of the radioactive material was more than 99.9% (NEN, Chicago). Previous experiments (Kuschinsky *et al.*, 1967a; Lüllmann, Peters & Van Zwieten, 1969) have shown that the glycosides are not metabolized and all radioactive material detected represents the original tritiated compound.

Unlabelled ouabain was dissolved in distilled water, unlabelled digitoxin in ethanol: water (70 : 30). The final alcohol concentration in the incubation medium did not exceed 0.1%, which in this system is biologically ineffective.

The composition of the Tyrode solution (mmol/litre) was as follows: NaCl 137.0, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, $NaHCO_3$ 12.0, NaH_2PO_4 0.21, glucose 5.5. The pH of the solution was adjusted to 7.35 by gassing with a mixture of 95% O_2 and 5% CO_2.

Results

Measurement of the size of the extracellular space

From previous experimental results (Lüllmann & Van Zwieten, 1967) the size of the extracellular space (ECS) of guinea-pig atria was estimated to be 0.35 ml/g wet weight. Atrial tissue, however, does not possess the well-developed transverse tubular system which is found in mammalian ventricular muscle (Sperelakis & Rubio, 1971). Therefore, the ECS in papillary muscle was determined since we expected to find the size of this compartment different from atrial tissue.

The uptake of [^{3}H]-sucrose (0.5×10^{-7} M) was measured after various periods of incubation and plotted as the tissue/medium radioactivity ratio (T/M ratio) against incubation time (Figure 1). A maximum T/M ratio of 0.54 was attained after 120 minutes. Uptake experiments with [^{3}H]-inulin yielded essentially the same results, i.e. the time course and the final T/M ratio were identical to those determined with [^{3}H]-sucrose. From these data, the size of 0.54 ml/g wet weight for the ECS of papillary muscle can be calculated (Kleinzeller & Knotkova, 1966).

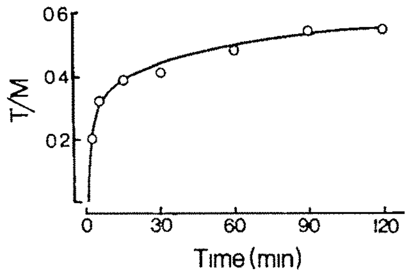

Figure 1 Time course of [^{3}H]-sucrose uptake by guinea-pig isolated papillary muscle. T/M = tissue/medium radioactivity ratio. The experimental points are mean values of at least 8 single determinations; standard error <5%. After 120 min, the maximum T/M value attained was 0.54.

Accumulation of [^{3}H]-ouabain at different concentrations of the drug in the incubation medium

The uptake of [^{3}H]-ouabain by ventricular muscle was investigated at different drug concentrations in the medium: 5×10^{-8} M, 1×10^{-7} M, 7.7×10^{-7} M, and 5×10^{-6} M. The time courses of the T/M ratios for these concentrations were compared in Figure 2. The uptake of the lowest concentration of ouabain (5×10^{-8} M) was not included in Figure 2 because its time course cannot be distinguished from the uptake of 1×10^{-7} M ouabain. The T/M ratios reached an equilibrium level which depended on the concentration studied. In the presence of ouabain, 1×10^{-7} M, the T/M ratio at equilibrium was 3.4. This value decreased to 1.8 and 0.82 respectively, when ouabain concentrations of 7.7×10^{-7} M and 5×10^{-6} M were used. In other words, at high concentrations of ouabain the T/M ratio at equilibrium level approached that of the ECS as established with [^{3}H]-sucrose. Furthermore, the time required to reach a steady tissue level of ouabain became shorter with increasing concentrations. With ouabain 1×10^{-7} M, the equilibrium level was attained after 120 min; in the presence of 7.7×10^{-7} M and 5×10^{-6} M, the times required decreased to 90 and 60 min respectively.

For an estimation of the amount of drug taken up by or bound to the cells only, it had to be assumed that ouabain was distributed in the ECS similarly to sucrose or inulin which seemed justified since the latter two compounds of different molecular size occupied a tissue space of equal size. Thus the uptake of ouabain in equilibrium was corrected for the amount of drug present in the ECS (Godfraind & Lesne, 1972) and plotted versus its concentration in the incubation medium (Figure 3a). This curve was found to fit a single Langmuir adsorption term:

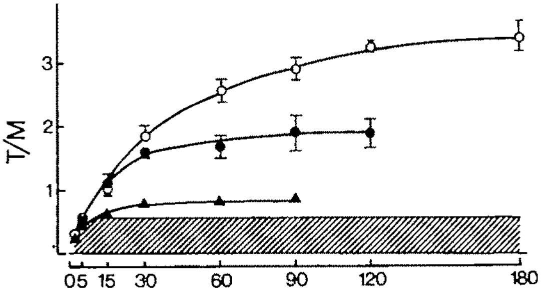

Figure 2 Time courses of the uptake by guinea-pig papillary muscle with a different concentration of [^{3}H]-ouabain in the incubation medium: 1×10^{-7} M (○), 7.7×10^{-7} M (●), and 5×10^{-6} M (▲). T/M = tissue/medium radioactivity ratio. The individual points represent mean values of at least 6 separate measurements. Vertical bars show s.e. mean. With increasing concentrations of ouabain, the T/M ratio approached the value of the extracellular space (hatched area). The time required to reach an equilibrium level decreased.

$$U = ax/x + K_a$$

where U = measured uptake, a = a constant describing the capacity of the adsorption site and K_a = equilibrium constant (Paton & Rang, 1965). On rearranging,

$$\frac{1}{U} = \frac{1}{a} + \frac{K_a}{a}\frac{1}{x}.$$

Plotting 1/uptake versus 1/concentration in the medium (Figure 3b) a straight line with a regression coefficient of $r = 0.997$ was obtained in the concentration range which is limited either by the specific activity of obtainable [^{3}H]-ouabain or by intoxication of the muscles. Within this biologically relevant range the uptake of ouabain by papillary muscles was a single saturable process. Furthermore, from the intercept of the straight line with the abscissa scale the equilibrium constant can be calculated. Thus the concentration of half maximal binding is 6×10^{-7} M. Similarly from the intercept with the ordinate scale the maximum number of binding sites could be estimated: it amounted to 1×10^{15}/g wet weight. This corresponds to a binding capacity of 1.9×10^{-6} mol ouabain/kg tissue. The number of binding sites occupied at various concentrations of ouabain during steady-state conditions (Table 1) can be calculated by correcting the total tissue content of ouabain for the amount present in the ECS as described above.

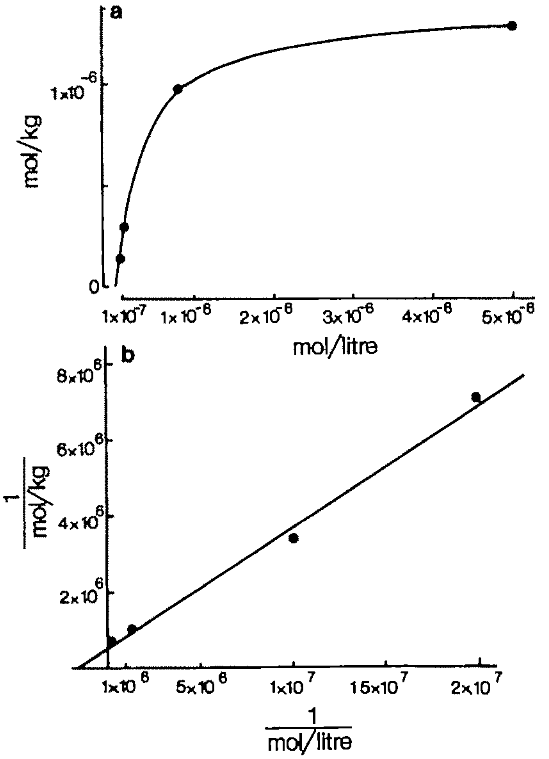

Figure 3 Uptake of $[^3H]$-ouabain by isolated papillary muscles of guinea-pig. (a) Ordinates: tissue content in mol/kg wet weight of tissue at equilibrium. Abscissae· ouabain concentration (in mol/l) in the perfusion medium. The uptake of ouabain was not directly proportional to its concentration in the perfusion fluid and could be described by a Langmuir adsorption term (b). (b) Reciprocal plot of the uptake. Ordinates: ouabain bound by the tissue (corrected for ouabain located in the ECS) as 1/mol/kg. Abscissae: concentration of ouabain in the incubation medium as 1/mol/litre. The linear regression coefficient of the straight line ($y = 0.32\,x + 5.3 \cdot 10^6$) equals $r = 0.997$.

Comparison of $[^3H]$-digitoxin and $[^3H]$-ouabain uptake

In ventricular tissue, digitoxin was accumulated to a much higher degree than ouabain which confirms the findings in atria. Figure 4 shows a comparison of the uptake of $[^3H]$-digitoxin and $[^3H]$-ouabain at 5×10^{-7} M and 7.7×10^{-7} M respectively. Digitoxin did not reach an equilibrium level during the 180 min period of observation. The T/M ratio for digitoxin uptake was 10 times higher than that for ouabain, which can only be explained by an intracellular accumulation of the hydrophobic digitoxin.

Influence of stimulation on the $[^3H]$-ouabain uptake

All experiments described so far were done with resting papillary muscles. However, it seemed important to investigate the influence of stimulation on the uptake of ouabain since mechanical and electrical activity might well be able to modify this process. Rhythmic contraction, for example, could facilitate the exchange of the ECS with the incubation medium and depolarization of the membrane might render it permeable to ouabain by some unknown mechanism. We therefore studied the ouabain uptake at 1×10^{-7} M in electrically driven papillary muscles. The results are presented in Figure 5. There was no difference between the uptake curves for stimulated and quiescent muscles, which indicates that the distribution of ouabain within the tissue must be essentially the same.

Exchange of tissue-bound ouabain with free ouabain in the medium

Previous studies in atria revealed a striking difference between the mere uptake of ouabain on

Table 1 Binding capacities for ouabain in ventricular and atrial muscle of guinea-pig

Tissue	*Ouabain concentration in the incubation medium (M)*	*T/M at equilibrium*	*Maximal* membrane-bound ouabain (mol/g wet wt.)*	*Occupied† binding sites per g wet wt.*
Papillary muscle	1×10^{-7}	3.4	3×10^{-10}	1.8×10^{14}
	7.7×10^{-7}	1.8	1×10^{-9}	6.0×10^{14}
	5×10^{-6}	0.82	1.4×10^{-9}	8.4×10^{14}
Atria‡	1.7×10^{-7}	0.68	4×10^{-11}	2.4×10^{13}

* Calculated from the total tissue content at equilibrium and corrected for the amount present in the ECS (see results section).

† Calculations based on Avogadro's number (6×10^{23}).

‡ Data taken from Kuschinsky *et al.*, 1968.

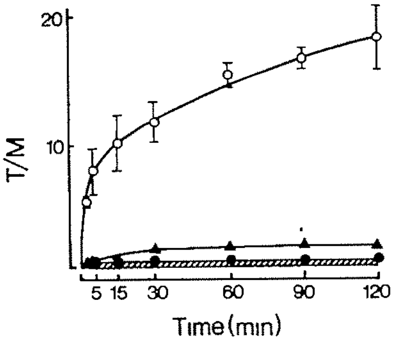

Figure 4 Comparison of the uptake of [^3H]-digitoxin (5×10^{-7} M) and [^3H]-ouabain (7.7×10^{-7} M) by isolated papillary muscles. Layout as in Figure 2. Digitoxin (○) was accumulated to a much greater extent in the tissue than ouabain (▲), and it did not reach an equilibrium level during the period of observation.

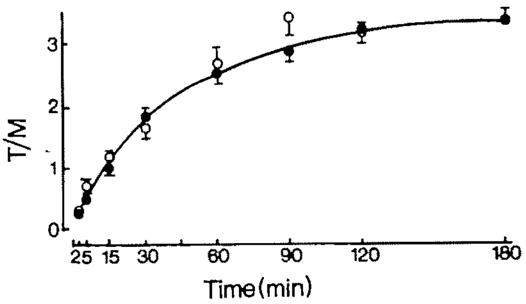

Figure 5 Uptake of [^3H]-ouabain (1×10^{-7} M) by quiescent (●) and stimulated (○) papillary muscles. Layout as in Figure 2. No difference in the rate of uptake could be found between quiescent and stimulated papillary muscles.

the one hand and the exchange of ouabain with previously bound, non-radioactive ouabain on the other hand. After preincubation with non-radioactive ouabain (0.5 μg/ml), the uptake of labelled drug proceeded more slowly (Kuschinsky *et al.*, 1968). In a similar experiment this 'exchange' of ouabain was studied in papillary muscles at a concentration of 1×10^{-7} M (Figure 6a). In order to ensure similar experimental conditions for all preparations in which either direct uptake or exchange were studied, the equilibration period for the control muscles was prolonged for an additional period of 180 min in normal Tyrode solution before determination of ouabain uptake. This was the same period of time required for equilibration with unlabelled ouabain in the exchange group. As illustrated in Figure 6a the differences between direct uptake and exchange were small but particularly obvious after 15 min of uptake or exchange.

The exchange of previously bound labelled ouabain with unlabelled ouabain was also studied at 1×10^{-7} M and compared with the corresponding wash-out curve (Figure 6b). The disappearance of radioactivity from the tissue occurred faster if unlabelled ouabain was present in the washing medium. No attempt was made to study the exchange of ouabain at higher concentrations, because contracture would develop during the long course of these experiments.

Wash-out curves for [^3H]-ouabain

In order to study the time courses of ouabain wash-out, the papillary muscles were preincubated with tritium-labelled ouabain until an equilibrium level was attained and then transferred into glycoside-free Tyrode solution for various periods of time. Again, the preparations were assayed for their content of radioactivity. The resulting wash-out curves for papillary muscles which had been preincubated at the concentrations 1×10^{-7} M, and 5×10^{-6} M are depicted in Figure 7a, where the ouabain content of the tissue is expressed as percent of the preload equilibrium level and plotted versus time in a semilogarithmic coordinate system. Analysis of the resulting curves revealed that initially ouabain was removed from the tissue at a higher rate than during the later phase of wash-out. In each case the experimental values could be fitted by a complex exponential curve: separating the two components by subtraction yielded two straight lines of different slopes. The initial rate of the wash-out was faster than the corresponding uptake; the late phase of the wash-out, however, occurred very slowly with a half life of 135 and 117 min for 1×10^{-7} M and 5×10^{-6} M ouabain respectively. Furthermore, this presentation of the data provides evidence that about 40%-60% of the tissue content of ouabain was released at a very slow rate and may therefore be bound rather tightly.

Figure 7b presents a similar plot of the time courses of the wash-out and of the exchange of labelled ouabain with unlabelled drug at 1×10^{-7} M. The graphical analysis of the data of the exchange experiments likewise revealed two phases of disappearance of radioactivity from the tissue. However, each phase was much faster than the corresponding wash-out phase. The half lives amounted to 3 and 65 min for the exchange as compared to 16 and 135 min respectively for the wash-out.

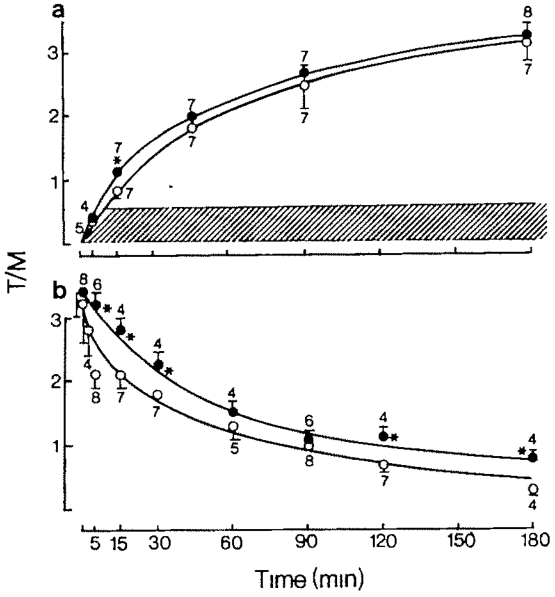

Figure 6 Comparison of (a) the uptake and 'exchange' and (b) the wash-out and 'exchange' of [^{3}H]-ouabain (1×10^{-7} M) in isolated quiescent papillary muscles. Lay-out as in Figure 2. The digits indicate the number of experiments for each point (a) The exchange (○) of active ouabain with inactive drug with which the muscles had been preloaded proceeded more slowly than the uptake (●) of radioactive ouabain. (b) The disappearance of radioactivity from the tissue was faster, if unlabelled drug was present in the washing medium.

* differences of experimental values statistically significant on a 5% level ($P < 0.05$).

Discussion

Recent investigations on the localization of tritiated cardiac glycosides in the heart muscle by means of autoradiography (Lõhr *et al.*, 1971) revealed that ouabain is mainly accumulated in the ECS and partly also at the plasma-membranes. Even after an 18 h incubation period during which marked intracellular oedema cannot be avoided, an intracellular location of glycoside close to the plasma-membrane is seen only occasionally and is very likely to be an artefact. The relatively small amount of ouabain found near the plasma-membrane, which would correspond to a 'membrane-bound' fraction, can readily be explained by the very high concentrations of ouabain (200 μg/l) used for these experiments. Due to its relatively small size, at high concentrations of ouabain the saturable compartment can

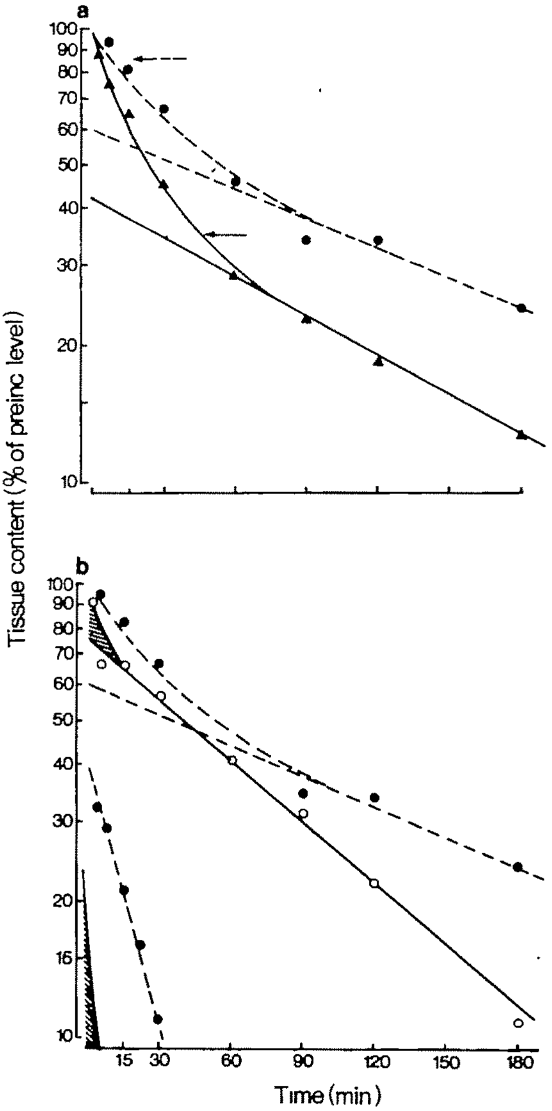

Figure 7 Semilogarithmic plots of the time courses of the wash-out and exchange of ouabain in isolated papillary muscle. (a) Time course of the wash-out [^{3}H]-ouabain after preincubation with 5×10^{-6} M (▲) and 1×10^{-7} M (●). Ordinates: ouabain tissue content expressed as percentage of the preincubation equilibrium level. The arrow indicates the relative size of the ECS at the preincubation equilibrium level: 1×10^{-7} M (dotted arrow) and 5×10^{-6} M (continuous arrow). For each wash-out curve, 2 separate phases could be distinguished. Neither wash-out curve yielded a straight line in the semi-logarithmic plot, i.e. they do not follow a simple exponential function. (b) Time courses of the wash-out of ouabain 1×10^{-7} M (●) and the 'exchange' from active to inactive ouabain (○). Lay-out as in (a). See text for further explanation.

hardly be separated from the drug present in the extracellular space.

In uptake experiments with different glycosides including ouabain, Dutta *et al.* (1968) found the major portion of their labelled material in a subcellular fraction which they assumed to be sarcoplasmic reticulum. Using similar homogenization procedures we could also find ouabain within a microsomal fraction. However, sucrose and inulin were traced in the same fraction (Lüllmann & Peters, 1974), which indicates that the lumen of the structure giving rise to the formation of microsomes during homogenization must have been continuous with the ECS. Therefore, it is more reasonable to assume that the prevailing structures of the microsomal fraction are plasma-membranes and sarcolemmal tubular systems rather than sarcoplasmic reticular membranes. The uptake experiments presented in this paper strongly suggest that in heart muscle there is a saturable compartment for ouabain (see also Figure 3). There are several possibilities for the localization of a saturable compartment. It could be located for example within the cell or it could represent either a saturable transport mechanism or an adsorption to the cell surface. We think our experimental data provide evidence for the latter possibility.

If it is assumed, for the sake of argument, that ouabain was accumulated at some membranous structure accessible only via the intracellular space, it follows that ouabain should have been present in the cell water before binding to this hypothetical site could occur. Furthermore, the bulk of cell water would be expected to be in equilibrium with the ECS and thus contain the same concentration of ouabain. This, however would be apparent as a further non-saturable compartment in the uptake kinetics. Our data provide no evidence for a compartment linearly dependent on the concentration in the medium. Due to its strong hydrophilic character ouabain does not penetrate the plasma-membrane unless transported by a specific mechanism. Such a transport mechanism, however, would be expected to transfer the drug from the ECS to some intracellular compartment until the latter is saturated. In such a model some drug would always be present intracellularly. Therefore, even at very high drug concentrations the T/M ratio could not attain a value below 1. With ouabain 5×10^{-6} M we found a considerably lower T/M ratio of 0.82; in other words, the T/M ratio was approaching the value of the ECS.

Digitoxin was accumulated to a much higher degree (almost 10 fold) than ouabain in papillary muscle. Furthermore, the uptake did not attain an equilibrium level after 3 h whereas with a similar concentration of ouabain the uptake had reached equilibrium after 90 min of incubation. We concluded that digitoxin must be taken up by the cells which is supported not only by the results of previous uptake experiments (Godfraind & Lesne, 1967; Kuschinsky *et al.*, 1968) but also by autoradiography studies (Waser, 1965; Lõhr *et al.*, 1971). Although ouabain and digitoxin exhibit such different distribution patterns, their pharmacological action is essentially the same. In fact, the plasma-membrane may be the site of action common to all cardiac glycosides; however, the uptake kinetics of hydrophobic glycosides are complicated by intracellular accumulation. This large 'unspecific' compartment makes it very difficult to estimate their membrane-bound fraction which should correspond to the membrane-bound fraction of ouabain and is probably responsible for its pharmacological effect (Kuschinsky *et al.*, 1968).

Kuschinsky *et al.*, (1967b) demonstrated that the uptake of [^{3}H]-digoxin by resting left auricles proceeds more slowly than by beating auricles. Thus the uptake process of digoxin seems to be related in some way to the excitation process of the membrane. It may well be possible that only the intracellular uptake of digoxin is facilitated by excitation, since the uptake of ouabain which cannot pass through the plasma-membrane into the intracellular space remains unaffected by the excitation process.

Comparing the result of our present investigation in papillary muscle with those found previously in atria (Kuschinsky *et al.*, 1967a, b, 1968; Kuschinsky & Van Zwieten, 1969) no qualitative though considerable quantitative differences could be demonstrated.

The ECS of atria was 0.35 ml/g wet weight (Lüllmann & Van Zwieten, 1967) whereas in papillary muscle an ECS of 0.54 ml/g wet weight was determined. The large size of the ECS in ventricular muscle was confirmed by estimations in the Langendorff preparation of the guinea-pig heart, where about 50% of the total tissue volume was found to represent this compartment (Peters, unpublished observation). The well-developed transverse tubular system in ventricular muscle (Sperelakis & Rubio, 1971) may account for this difference in the size of the ECS.

In order to compare the binding capacities for ouabain in ventricular and atrial muscle, we calculated the number of binding sites occupied at equilibrium level for different concentrations of ouabain and listed these values together with the estimate for atria based upon the data of Kuschinsky *et al.* (1968) in Table 1. Although the T/M ratios established for the uptake of different concentrations of ouabain in atria varied within such a small range (0.5-0.7) that no significant concentration-dependent differences could be measured, the maximum number of binding sites could be roughly estimated. It amounted to approximately 2×10^{13} binding sites/g wet weight. This value is only 1/40 of the number calculated for ventricular tissue, i.e. 8×10^{14} binding sites/g wet weight. These estimates are supported by the results of Rieger & Kuschinsky (1972) who also found a greater accumulation of glycoside in the ventricle than in atria. Some of the results reported in this paper are not in agreement with the findings of Godfraind & Lesne (1972) in atria, which may be explained by the different concentration ranges investigated.

The differences in the time courses of the exchange and wash-out experiments (Figure 7b) require some further considerations. Since our uptake kinetics revealed a single saturable compartment the two phases of ouabain wash-out cannot be explained by an unsaturable intracellular compartment (Marks, 1972). We have rather to assume two sets of binding sites with similar association constants but different dissociation constants, i.e. 'fast' and 'slow' binding sites.

If a labelled molecule of ouabain dissociates from a 'slow' binding site, it has a reasonable chance to be reassociated to either a 'fast' or a 'slow' binding site when no other glycoside is present in the medium. This rebinding will delay the disappearance of radioactivity from the tissue during wash-out. However, if unlabelled ouabain is present in the medium and therefore also in the ECS, the concentration of dissociating labelled molecules will be diluted in the vast reservoir of inactive molecules. This in turn diminishes their chances of reassociation considerably and thus radioactivity will disappear faster from the tissue. For these reasons, the second slow phase of exchange of active to inactive drug will give a better estimate of the rate of dissociation from the binding sites than the wash-out curve. Thus the half life of dissociation from the 'slow' binding sites is more likely to be 65 min than 135 minutes.

The following numerical example is an attempt to explain the differences in the time courses of uptake and exchange of ouabain (Figure 6a) assuming a half life of 65 min for the slowly dissociating binding sites. During the wash-out of ouabain 1×10^{-7} M (Figure 7) almost 60% of all binding sites are slowly dissociating. Thus, in the beginning only fast dissociating bindings sites (i.e. 40% of all binding sites) can take part in the exchange. With time, an increasing fraction of slowly dissociating binding sites is also involved: after 1 half life (65 min) half of them resulting in a total of 70% of available binding sites, after 3 half lives (195 min) 9/10 of them, rendering a total of 94% of the binding sites available for exchange. This rough estimate may explain why the delay in the uptake of radioactivity after preincubation with inactive ouabain is most prominent during the early phases of exchange (Figure 6a) when a relatively large number of the binding sites available under these conditions is still occupied by inactive drug which dissociates slowly.

From our data presented in this paper we can conclude that in papillary muscle there must be a saturable compartment which clearly can be distinguished from the ECS. Its filling is associated with the binding of ouabain to some structure located probably at the plasma-membrane and including the tubular system. The time course of dissociation of ouabain from the binding sites suggests that they can exhibit two different kinetic properties. The significance of these membrane located binding sites for the site of action for the positive inotropic effect is still under investigation.

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EVIDENCE THAT ADRENERGIC NERVES ARE RESPONSIBLE FOR THE ACTIVE UPTAKE OF NORADRENALINE IN THE GUINEA-PIG ISOLATED TRACHEA

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1 6-Hydroxydopamine (50 mg/kg, i.p.) was given to guinea-pigs to destroy the adrenergic nerve terminals in the trachea.

2 The destruction was demonstrated by fluorescence histochemistry, which showed a marked loss of beaded fluorescent terminal fibres and by electrical transmural stimulation of the isolated atropinized trachea, which showed a marked reduction of dilator responses.

3 Such tracheae showed greatly reduced uptake-with-retention of (—)-[^{3}H]-noradrenaline in incubation experiments and the efflux curve of radioactive material showed a selective but incomplete reduction in the volume of the slowly exchanging compartment.

4 It is concluded that much, but perhaps not all, of the uptake-with-retention occurs into adrenergic nerves.

Introduction

Foster & O'Donnell (1972) examined the properties of the uptake-with-retention of radioactive material occurring when the guinea-pig trachea was exposed to (—)-[^{3}H]-noradrenaline. Though lacking direct evidence, they concluded that the uptake occurred into adrenergic nerves since (a) adrenergic nerves can be demonstrated in the guinea-pig trachea pharmacologically (Foster, 1964) and histochemically (O'Donnell & Saar, 1973) and (b) the properties of the uptake resembled those of established intraneuronal uptake of noradrenaline, namely, sodium dependence, susceptibility to ouabain, saturability, energy dependence and susceptibility to inhibition by cocaine and desipramine (cf. Iversen, 1970).

The experiments described in this paper were carried out in an attempt to obtain more direct evidence that the radioactive material firmly retained in trachea after incubation in (—)-[^{3}H]-noradrenaline 50 nM had been taken up into adrenergic nerves. The approach adopted used 6-hydroxydopamine (6-OHDA) as a tool to destroy the functional integrity of the adrenergic nerve terminals in the trachea (O'Donnell & Saar, 1974). The uptake-with-retention in tracheae from animals pretreated with 6-OHDA was compared with that in controls and the effectiveness of the destruction was checked both histochemically, by the fluorescence technique of Falck & Hillarp (Falck, 1962), and pharmacologically, by transmural stimulation to excite adrenergic nerves and so elicit relaxant responses.

Methods

Female guinea-pigs (250-560 g) were used. A dose of 50 mg/kg 6-OHDA was given intraperitoneally to 12 animals 24 h before examining the trachea. Control animals, matched with these for body weight, received 0.9% w/v NaCl solution (saline).

Fluorescence histochemistry and uptake-with-retention

The laryngeal ring from each of six control animals and six animals pretreated with 6-OHDA was taken for fluorescence histochemistry as described by O'Donnell & Saar (1973). Before freeze-drying the rings were incubated in α-methylnoradrenaline 1 μM for 30 minutes. The remainder of the trachea was cut into rings and the uptake-with-retention of radioactive material measured according to the method of Foster & O'Donnell (1972) by incubating for 15 min in (—)-[^{3}H]-noradrenaline 50 nM (and ascorbic acid 0.6 mM) and washing for

30 min to clear the interstitial fluid of radioactive material.

Transmural stimulation and efflux

Each trachea from six control and six 6-OHDA pretreated guinea-pigs was set up, in Krebs solution containing atropine (350 nM), for transmural stimulation essentially according to the method of Foster (1964), although changes in intraluminal pressure, rather than volume, were recorded (Farmer & Coleman, 1970). A 20 min equilibration period at atmospheric pressure was allowed, plus a further 10 min after closing the system. Each trachea was transmurally stimulated (positive electrode in lumen) with 40 mA pulses, width 0.5 ms for 7 seconds. Relaxant responses were produced by three stimulation trains at each of 1.25, 2.5 and 5 Hz given at 4 min intervals and then by three trains at each of 10 and 20 Hz given at 5 min intervals. The response to transmural stimulation was measured as the mean change in intraluminal pressure obtained with the second and third trains of stimuli in each sequence. The trachea was then cut into rings and allowed to equilibrate in fresh Krebs solution containing atropine (350 nM) at 37°C for 1 h before transfer to 4 ml of incubation medium also containing (–)-[^{3}H]-noradrenaline (50 nM) and ascorbic acid (0.6 mM) for 15 minutes. The efflux of radioactive material on repeated washing of tissue was then measured, essentially by the method described by Foster & O'Donnell (1972) but following it for the longer time of 75 minutes.

Radioassay

Samples (1 ml) of washings or of the clear supernatant of a 0.4 N perchloric acid homogenate of trachea were added to 10 ml of phosphor, which had the composition: 333 ml Triton-X 100; 5.5 g PPO (2,5-diphenyl-oxazole); 0.1 g dimethyl-POPOP (1,4-di-(2(4-methyl-5-phenyl oxazolyl))-benzene); toluene (A.R.) to 1 litre. The automatic external standard channels ratio method was used to correct for quenching.

Drugs

6-OHDA was obtained from Hassle, Sweden. Doses are given in terms of the hydrochloride. (–)-Noradrenaline-[7-^{3}H] (5.6 Ci/mmol, radiochemical purity greater than 97%) was obtained from the Radiochemical Centre, Amersham. Other drugs and chemicals were from commercial sources.

Statistical analysis

The measure of variation of the mean quoted is the standard error. Student's *t*-test (2 tailed) was used to assess the probability that two means did not differ. Correlation was examined by the non-parametric Kendall rank correlation coefficient (Siegel, 1956).

Results

The mean tracheal content of radioactive material after 15 min incubation with (–)-[^{3}H]-noradrenaline and 30 min washing was 70.6 ± 12.3 pmol/g in the control group, while in the 6-OHDA pretreated group it was 20.1 ± 2.4 pmol/g ($P < 0.005$).

Beaded, terminal fibres were seen in the sections from five of six control tracheae but were absent from the sections of one, which also gave the smallest uptake-with-retention (38.5 pmol/gram). Beaded terminal fibres were absent from sections from five of the 6-OHDA pretreated tracheae but two or three faint beaded strands were present in one, which also gave the largest uptake-with-retention (30.5 pmol/gram). The probability (P) that this combination, or one more extreme, would arise by chance is 0.04 (Fisher's exact test).

The mean dilator response to each frequency of stimulation was significantly smaller in tracheae from 6-OHDA-treated animals than in the control tracheae, indeed at the lower three frequencies the mean response in the 6-OHDA group did not differ significantly from zero (Figure 1).

In the efflux experiments the tracheal content of radioactive material at all times after incubation was lower in each of the 6-OHDA pretreated group than in any of the controls. Curve stripping (Riggs, 1963) revealed three exponential components to the decay of tissue radioactivity with time in both groups (Table 1). The effect of 6-OHDA was selectively directed to the most slowly exchanging compartment, whose apparent volume (C) was reduced while its efflux rate constant (γ) was increased.

The fall in intraluminal pressure caused by transmural stimulation at 5 Hz and the size of this slowly exchanging compartment estimated as the intercept (C) at the beginning of washout seemed to be related to each other (Figure 2). The estimated volume of distribution of slowly exchanging radioactive material at zero response was 0.7 ml/g which corresponds with an uptake-with-retention (30 min washing) of 23 pmol/gram.

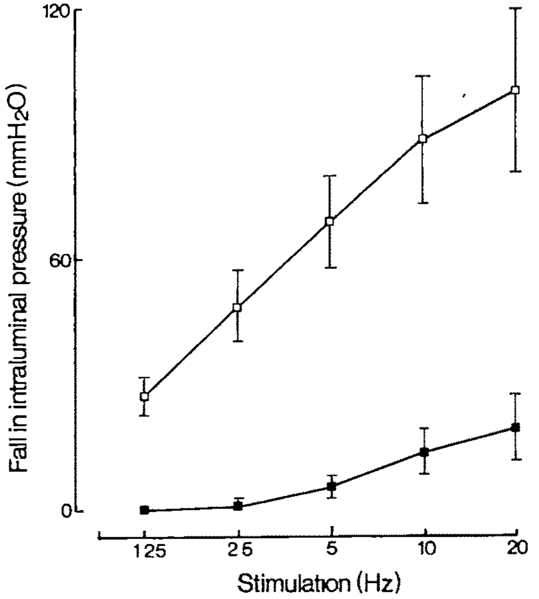

Figure 1 Effect of 6-hydroxydopamine (6-OHDA) pretreatment on the dilator response to transmural stimulation of atropinized trachea. Ordinates — mean fall in intraluminal pressure (mm water). Vertical bars show s.e. mean. Abscissae — frequency of stimulation (Hz) applied for 7 seconds. (□) Control, (■) 6-OHDA pretreated trachea.

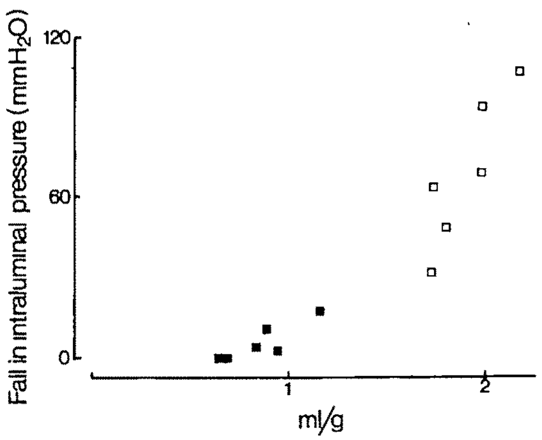

Figure 2 The relationship between response to transmural stimulation and tissue radioactivity in atropinized tracheae. Ordinates — mean fall in intraluminal pressure (mm water), obtained with transmural stimulation at 5 Hz for 7 seconds. Abscissae — apparent volume of slowly exchanging compartment of tissue radioactivity after incubation with 50 nM (—)-[^{3}H]-noradrenaline for 15 minutes. (□) Control tracheae correlate with $P < 0.05$; (■) 6-OHDA pretreated tracheae correlate with $P < 0.1$. All twelve tissues correlate with $P < 0.001$

Table 1 Separation of exponents from the efflux curve $Y_t = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-\gamma t}$, where Y_t = radioactive material content of 1 g of trachea, t = time of washing

Exponential decay	*Control*	*6-OHDA*	*2P*
,Very fast			
A (ml/g)	1.24 ± 0.10	1.41 ± 0.16	0.6
α (min^{-1})	1.083 ± 0.073	1.158 ± 0.122	0 6
Fast			
B (ml/g)	0.87 ± 0.01	1.07 ± 0.08	0.06
β (min^{-1})	0.104 ± 0.003	0.118 ± 0.008	0.15
Slow			
C (ml/g)	1.90 ± 0.07	0.85 ± 0.08	<0.0001
γ (min^{-1})	0.0055 ± 0.003	0.0158 ± 0.0019	0.003

The very fast exponential decay ($t_{\frac{1}{2}}$ c 0.6 min) probably represents radioactive incubation medium adhering to the tissue on transfer to the first washing tube. The fast phase ($t_{\frac{1}{2}}$ c 6 min) represents radioactive material in the interstitial space of the tissue and the slow one ($t_{\frac{1}{2}}$ c 120 min) 'cellular' radioactivity (Foster & O'Donnell, 1972).

Discussion

The primary objective of this study was to obtain evidence that the radioactive material which was firmly retained in the isolated trachea after exposure to a low concentration of (—)-[^{3}H]-noradrenaline had been taken up into adrenergic nerves. This objective was attained. Pretreatment with 6-OHDA was demonstrated both histochemically and by transmural stimulation to cause loss of the functional integrity of adrenergic nerves of the trachea. Moreover, preparations so treated retained significantly less radioactive material than controls after incubation with 50 nM (–)-[^{3}H]-noradrenaline.

There is evidence that 6-OHDA was not completely effective in destroying all adrenergic nerves in that (a) beaded fluorescent fibres were seen in one pretreated trachea, (b) dilator responses to the higher frequencies of transmural stimulation were reduced but not abolished, (c) the uptake-with-retention of radioactive material was reduced but not abolished, and (d) the apparent volume of the most slowly exchanging compartment of tissue radioactivity, which is its basis, was reduced but not to zero. It is a matter for speculation whether the residual dilator responses to transmural stimulation were mediated by residual adrenergic nerves or by non-adrenergic nerves (Coleman, 1973). However O'Donnell & Saar (1974) have shown that higher doses of 6-OHDA always cause loss of beaded fluorescent fibres from the trachea, but do not further reduce its uptake-with-retention of radioactive material.

However the question arises does all the uptake-with-retention represent radioactive material taken up into adrenergic nerves. None of the treatments shown in previous publications to inhibit significantly uptake-with-retention of radioactivity has abolished it; a residuum varying from approximately 10 pmol/g (ouabain, Foster & O'Donnell, 1972; and guanethidine, Foster, 1968) to 17 pmol/g (phenoxybenzamine, Foster, 1968) has always remained despite treatment with apparently maximally effective concentrations of inhibitors of uptake.

O'Donnell & Saar (1973) have suggested, on the basis of histochemical evidence, that after incubation with a high concentration of noradrenaline the noradrenaline which is bound to cartilage (extraneuronal, extracellular) does not wash away quickly enough to be dissipated in 30 minutes. Experiments to test the idea that this is the origin of the residuum are in progress.

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EFFECT OF ANTI-INFLAMMATORY DRUGS ON THE MEMBRANE POTENTIAL OF VASCULAR ENDOTHELIAL CELLS *in vitro*

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1 Cells of the aortic endothelium isolated from the guinea-pig and bathed at 37° C with a calcium-free superfusion fluid had membrane potentials of −41 ± 7 mV (mean ± s.e. mean).

2 Depolarization was produced by addition of potassium (50-200 mM) or certain other monovalent metal cations to the superfusion fluid. Depolarization was rapidly reversed on return to the original superfusate.

3 Several divalent metal cations, notably calcium (16 mM), caused depolarization which was only slowly and incompletely reversed on return to the original calcium-free superfusate.

4 Repolarization after exposure to calcium was accelerated and made more complete by addition of indomethacin (0.25 mM) to the superfusate.

5 The trivalent cations of lanthanum, aluminium or iron (0.1 mM) inhibited the depolarizing effect of calcium (16 mM).

6 Exposure to histamine (100 μg/ml) or heating to 45° C for 1 h caused depolarization in the presence but not in the absence of calcium. Subsequent removal of histamine or cooling again to 37° C in the continued presence of calcium permitted only slow and partial repolarization. However, repolarization was more rapid and complete in the presence of indomethacin (0.25 mM).

7 Heating to 45° C for 5 h in the presence of calcium caused progressive and almost complete depolarization. Lanthanum, cinchocaine, indomethacin, flufenamic, meclofenamic and salicylic acids, phenylbutazone and aminopyrine each reduced the depolarization, but hydrocortisone, chloroquine, benzindamine, isoprenaline and aminophylline did not.

Introduction

Indomethacin and some pharmacologically related drugs reduce the uptake of calcium by electrically stimulated smooth muscle (Northover, 1972). Adenosine triphosphate-dependent binding of calcium to microsomes derived from smooth muscle or vascular endothelium is also inhibited by these drugs (Northover, 1973). Since the binding of calcium to cellular membranes alters their electrical properties (Bianchi, 1968; Bülbring and Tomita, 1970) the influence of anti-inflammatory drugs on the electrical activity of vascular endothelial cells was of interest. This paper describes the inter-relationships between the effects of anti-inflammatory drugs and of the concentration of calcium in the bathing fluid on the membrane potential of endothelial cells *in vitro*.

Methods

Albino guinea-pigs weighing 500-800 g were killed by a blow on the head. The thoracic aorta was removed immediately after death and opened longitudinally with fine scissors. A rectangular sheet of the vessel wall was tied, with its endothelial face upwards, to the bottom of a trough 10 cm long, 1 cm wide and 1 cm deep, made of Perspex. A thermostatically regulated water jacket around the trough enabled its contents to be kept at any selected temperature between ambient and 50° C (37° C unless otherwise specified). Superfusion fluid was warmed and delivered to one end of the trough at 10 ml/min by means of a roller pump. Fluid was withdrawn by suction through a constant level device from the other end. The contents of the trough were kept mixed by means of a stream of air bubbles. The composition of the basic superfusion fluid (BSF) was (mM): NaCl 130; KCl 6; glucose 11; $MgCl_2$ 2; tris-(hydroxymethyl)-methylamine 10, adjusted to pH 7.4 with acetic acid.

The mesothelium of the jejunal mesentery and the endothelium of the abdominal vena cava and of the hepatic portal vein of the guinea-pig were used in place of the aorta in some experiments.

Membrane potentials were recorded by means of glass pipettes filled by boiling for 3 min at reduced pressure with freshly filtered 3 M KCl solution. Each pipette contained a Ag/AgCl wire electrode which was connected to a single-ended cathode follower circuit (Electrophysiological Instruments). The output from the cathode follower was led both to an oscilloscope and to a pen-recorder. No action potentials were seen on the oscilloscope screen throughout these experiments and all measurements of membrane potential were made from the pen-recorder trace.

One end of a glass tube filled with 3 M KCl solution gelled with 3% agar was dipped into the superfusate in the trough. The other end contained a Ag/AgCl wire electrode which was earthed via a $10^2\ \Omega$ resistor. At intervals throughout each experiment current was passed to earth through this resistor and the voltage generated between the superfusate and earth used to calibrate the recording and to determine the resistance of the pipettes. Pipettes were retained for further use only if their resistance continued to be approximately $10^7\ \Omega$. In preliminary experiments the tip potential of 14 pipettes with this resistance was measured by the method of Adrian (1956) and found to be between 1 and 5 mV.

Pipettes were lowered vertically towards the tissue with a micromanipulator (Narishege, type MM3). A recording was considered to begin with the first negative change of voltage encountered, which was assumed to originate from the interior of a member of the surface layer of cells. The pipette was not moved for 50 s and was then withdrawn into the superfusate. The difference in voltage recorded just before and just after withdrawal was taken as the membrane potential. A recording was rejected as invalid only if the pipette failed to register the same potential (±1 mV) after withdrawal from the cell as it had done before entering it. No attempt was made to select cells with a particularly high or stable membrane potential. Membrane potentials registered at 10 places in the tissue were averaged and further groups of 10 such recordings taken at 15 or 60 min intervals throughout the experiment. To reduce vibration the superfusion trough and the micromanipulator were mounted on a heavy steel plate resting on 3 hollow rubber hemispheres.

Substances to be tested were freshly prepared as concentrated solutions which were then slowly delivered from a syringe-pump into the superfusate in the trough, the contents of which were kept mixed by a stream of air bubbles. Histamine dihydrochloride, acetylcholine bromide, (±)-isoprenaline sulphate, (−)-noradrenaline acid tartrate, cinchocaine hydrochloride, benzindamine hydrochloride and chloroquine hydrochloride were dissolved in distilled water and the solutions adjusted to pH 7.4 with NaOH solution. Concentrations refer to the free bases. Indomethacin, phenylbutazone, 1,2-bis-2-aminoethoxyethane-$NNN'N'$-tetraacetic acid (EGTA), phenylacetic acid, salicylic acid, flufenamic acid, meclofenamic acid and ibuprofen were dissolved in water with the aid of a slight excess of Na_2CO_3, and then rapidly adjusted to pH 7.4 with acetic acid solution. Concentrations refer to the free acids. Hydrocortisone sodium succinate was dissolved in distilled water to give a solution of pH 7.4. Concentration refers to the free alcohol. Aminophylline and aminopyrine were dissolved in distilled water and adjusted to pH 7.4 with acetic acid solution. The pH of the superfusate leaving the trough was recorded continuously to ensure that it remained at pH 7.4 ± 0.1.

Results

Aortic endothelium

Membrane potential in the presence of various metal cations The mean of 860 recordings of the membrane potential of cells in contact with the BSF was 41.1 ± 7.2 mV, with the inside negative. Addition of KCl to the superfusing fluid, with or without the addition of calcium 1 mM, caused a concentration-dependent depolarization (Figure 1). When the concentration of KCl was reduced to 6 mM again the cells promptly repolarized (Figure 2). Rubidium and caesium chlorides exerted similar actions to that of KCl, whereas the chlorides of sodium and lithium in concentrations up to 100 mM had no significant effect upon the membrane potential (Table 1). The tonicity of the bathing fluid was allowed to rise as a result of the addition of KCl. However, depolarization seen with KCl was presumably not due to the hypertonicity of the bathing fluid, since addition of similar concentrations of the chlorides of sodium and lithium failed to depolarize the cells.

Addition to the BSF of the chlorides of a number of divalent metal cations, including calcium, caused concentration-dependent depolarization (Table 1). In contrast to the effects of the monovalent metal cations the action of the divalent metal cations was only slowly and incompletely reversed by washing the tissue with the BSF (Figure 2 and Table 1). Reversal of the effect of calcium on returning to the BSF was accelerated by the presence of indomethacin (Figure 3). Magnesium was the only divalent metal ion tested which lacked a statistically significant depolarizing

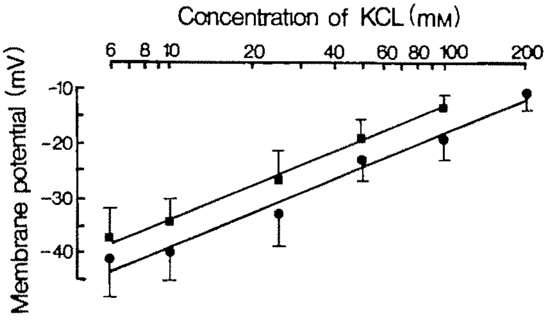

Figure 1 Membrane potential of aortic endothelial cells after exposure for 30 min to various concentrations of KCl in the absence of calcium (•) or in the presence of calcium 1 mM (▪). Each point is the mean of 40 observations. Vertical bars show s.e. mean.

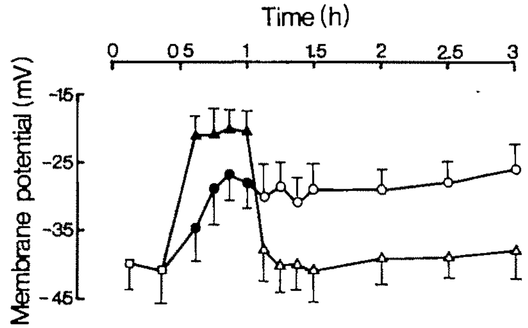

Figure 2 Membrane potential of aortic endothelial cells in the presence of 100 mM KCl (▲), 16 mM $CaCl_2$ (•) or the basic superfusion fluid (open symbols). Each point is the mean of 30 or 60 observations. Vertical bars show s.e mean.

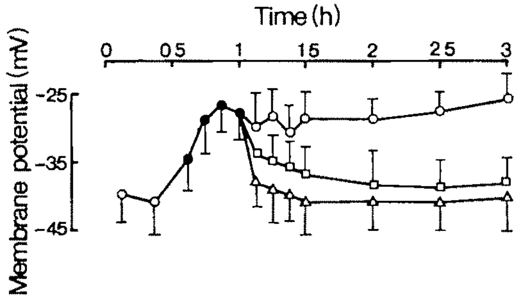

Figure 3 Membrane potential of aortic endothelial cells in the basic superfusion medium (○) or in the basic superfusion fluid plus 16 mM $CaCl_2$ (•), 0.25 mM indomethacin (□) or 0.1 mM $LaCl_3$ (△). In those experiments where indomethacin or lanthanum were added they were present in the superfusate from time 1 h onwards. Each point is the mean of 30 or 90 observations. Vertical bars show s.e. mean.

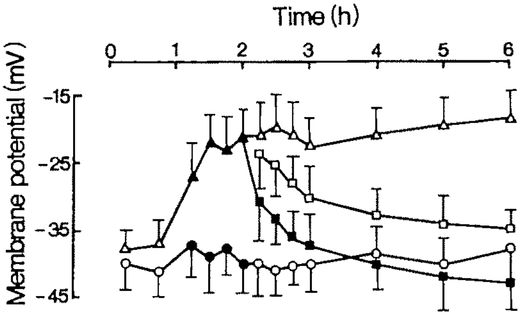

Figure 4 Membrane potential of aortic endothelial cells in the basic superfusion fluid (○ or •) or in a superfusate containing in addition 1 mM $CaCl_2$ (△ or ▲), 0.1 mM EGTA (▪) or 1.0 mM $CaCl_2$ plus 0.25 mM indomethacin (□). In those experiments where EGTA or indomethacin were added they were present in the superfusate from time 2 h onwards. Histamine (100 µg/ml) was infused from time 1 h to time 2 h (closed symbols). Each point is the mean of 30 or 90 observations. Vertical bars show s.e. mean.

action when added to the BSF in a final concentration of 16 mM (Table 1). It can be seen from Table 1 that the membrane potential of endothelial cells bathed with the BSF was not significantly different from that of cells bathed with a fluid containing no added magnesium. Moreover, the addition of magnesium 16 mM to a previously magnesium-free superfusion fluid caused no significant change in the recorded membrane potential of the endothelial cells whether or not the bathing fluid contained calcium (Table 1). Thus, magnesium not only lacked a calcium-like action but it also lacked a calcium-antagonizing action.

The chlorides of the trivalent metal cations of lanthanum, aluminium and iron failed to exert a statistically significant effect upon the membrane potential of endothelial cells when added to the BSF in a concentration of 0.1 mM, as shown in Table 1. Higher concentrations were not employed because they caused precipitation. Each of the three trivalent cations at a concentration of 0.1 M reduced the depolarizing effect of calcium 16 mM (Table 1). Like indomethacin, lanthanum accelerated the repolarization of cells which had been exposed to calcium previously (Figure 3).

Membrane potential in the presence of histamine Histamine rapidly depolarized endothelial cells in the presence but not in the absence of calcium (Figure 4). The concentration of histamine required to produce a given degree of depolarization was reduced by increasing the concentration of calcium between 0.1 and 10 mM. Thus, histamine enhanced the depolarizing effect of calcium rather than causing depolarization itself. In the presence of calcium 1 mM neither acetylcholine nor noradrenaline altered the membrane potential when tested in concentrations up to 1 mg/ml.

Figure 4 shows that when the aorta was transferred, after treatment with histamine plus calcium, to a superfusate containing 0.1 mM EGTA but no added calcium, repolarization was rapid and complete. In contrast, discontinuation of histamine but continuation of the presence of calcium caused slow and incomplete repolarization. However, the endothelial cells repolarized after histamine had been discontinued and despite the continued presence of calcium, when indomethacin was added to the superfusate. Thus, the action of indomethacin in the presence of calcium resembles the effect of removing calcium with EGTA.

Table 1 Effect of various cations on the membrane potential of aortic endothelial cells

		*Membrane potential (mV negative)**						
Cation†	*Concentration (mM)*	*During exposure to cation*	*During washout of cation with the BSF. Duration (min)*					
			10	*20*	*30*	*40*	*50*	*60*
BSF	–	41 ± 6‡	40 ± 5	38 ± 5	42 ± 6	39 ± 4	40 ± 5	37 ± 4
BSF, magnesium-free §	–	39 ± 5	39 ± 6	37 ± 5	38 ± 5	36 ± 5	35 ± 6	34 ± 7
		Monovalent cations						
Potassium	100	20 ± 3π	29 ± 4	36 ± 5	40 ± 6	39 ± 6	41 ± 5	38 ± 7
Rubidium	100	22 ± 4π	31 ± 3	39 ± 5	37 ± 6	38 ± 5	42 ± 7	40 ± 5
Caesium	100	18 ± 3π	28 ± 4	35 ± 6	39 ± 5	36 ± 5	40 ± 6	42 ± 7
Lithium	100	37 ± 6	39 ± 7	41 ± 6	40 ± 7	40 ± 6	39 ± 8	41 ± 5
		Divalent cations						
Magnesium §	16	34 ± 6	38 ± 5	37 ± 7	39 ± 6	36 ± 5	39 ± 6	38 ± 6
Magnesium′	16	34 ± 7	35 ± 5	35 ± 4	32 ± 5	33 ± 5	32 ± 4	32 ± 4
Calcium	16	26 ± 4π	28 ± 3	29 ± 4	27 ± 5	25 ± 4	27 ± 5	26 ± 4
Calcium §	16	25 ± 5π	26 ± 5	25 ± 7	28 ± 4	24 ± 5	26 ± 3	25 ± 6
Calcium	8	28 ± 5π	29 ± 5	31 ± 4	32 ± 5	30 ± 6	29 ± 5	31 ± 4
Calcium	4	31 ± 6	32 ± 5	33 ± 5	30 ± 6	34 ± 5	31 ± 7	32 ± 5
Calcium	2	32 ± 5	32 ± 6	34 ± 6	35 ± 5	33 ± 6	36 ± 6	37 ± 7
Barium	16	27 ± 4π	26 ± 6	29 ± 5	31 ± 5	30 ± 6	31 ± 5	29 ± 6
Strontium	16	29 ± 4π	30 ± 4	28 ± 5	31 ± 6	30 ± 5	29 ± 4	28 ± 5
Manganese	16	21 ± 3π	18 ± 3	29 ± 4	32 ± 5	33 ± 4	35 ± 4	34 ± 5
Zinc	16	20 ± 3π	19 ± 4	25 ± 3	34 ± 5	36 ± 4	32 ± 5	35 ± 6
Cobalt	16	18 ± 4π	15 ± 3	17 ± 4	21 ± 3	22 ± 4	26 ± 5	26 ± 6
		Trivalent cations						
Lanthanum	0.1	43 ± 5	37 ± 6	38 ± 5	41 ± 6	42 ± 5	40 ± 7	41 ± 6
Aluminium	0.1	42 ± 6	40 ± 5	41 ± 6	43 ± 6	40 ± 7	39 ± 7	38 ± 6
Iron′	0.1	43 ± 5	36 ± 6	39 ± 5	40 ± 5	42 ± 6	40 ± 7	38 ± 5
Lanthanum Calcium	0.1 16	39 ± 5	39 ± 6	42 ± 4	40 ± 7	39 ± 6	43 ± 5	40 ± 6
Aluminium Calcium	0.1 16	38 ± 5	39 ± 7	38 ± 4	37 ± 5	39 ± 6	38 ± 4	37 ± 5
Iron Calcium	0.1 16	34 ± 6	37 ± 5	39 ± 6	35 ± 5	35 ± 7	34 ± 4	35 ± 6

† The chloride salt of the cation added to the BSF for 30 minutes. § In these experiments the BSF was made up without $MgCl_2$. ′ In these experiments the BSF was made up without $MgCl_2$ but with $CaCl_2$ 2 mM added. * The mean of at least 30 observations ± s.e. mean.

‡ A significant difference exists between the value marked ‡ and the values marked π (Student's *t* test, $P < 0.05$).

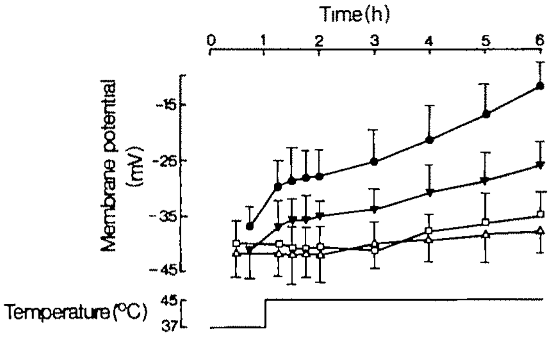

Figure 5 Membrane potential of aortic endothelial cells heated to 45° C. Potentials recorded in the basic superfusion fluid (▾) or in a superfusate containing in addition 1.0 mM $CaCl_2$ (●), 0 1 mM EGTA (■) or 1.0 mM $CaCl_2$ plus 0.1 mM $LaCl_3$ (△). Each point is the mean of 30 observations. Vertical bars show s.e. mean.

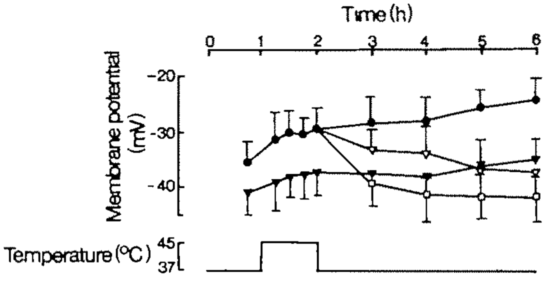

Figure 6 Membrane potential of aortic endothelial cells. The endothelium was heated to 45° C for 1 hour. Potentials were recorded in the basic superfusion fluid (▾) or in a superfusate containing in addition 1.0 mM $CaCl_2$ (●), 0.1 mM EGTA (■) or 1.0 mM $CaCl_2$ plus 0.25 mM indomethacin (□). In those experiments where EGTA or indomethacin were added they were present in the superfusate from time 2 h onwards. Each point is the mean of 30 or 90 observations. Vertical bars show s e. mean.

Membrane potential at elevated temperatures Exposure of the endothelium to the BSF at 45° C for up to 5 h caused progressive depolarization which was enhanced by the addition of calcium 1 mM (Figure 5). Addition of EGTA 0.1 mM to the BSF protected against this depolarization (Figure 5). A temperature of 45° C was chosen for these experiments because preliminary experiments had shown that in the presence of calcium 1 mM lower temperatures caused only inconsistent depolarization. Heating to 50° C, on the other hand, caused depolarization more rapidly than at 45° C but the maximum depolarization produced was no greater than at 45° C.

In some experiments (Figure 6) aortas which had been heated to 45° C for 1 h in the presence of calcium 1 mM were cooled again to 37° C. In the continued presence of calcium the tissue remained depolarized, but when the tissue was exposed to the BSF some repolarization occurred. When the tissue was exposed after cooling to a superfusate containing EGTA the repolarization was almost complete (Figure 6). Even in the continued presence of calcium 1 mM the presence of indomethacin permitted considerable repolarization (Figure 6).

The depolarizing effect of prolonged exposure to a temperature of 45° C in the presence of calcium 1 mM was reduced by addition of lanthanum or cinchocaine to the superfusate (Figure 5 and Table 2). Anti-inflammatory drugs with a lanthanum-like action included indomethacin, ibuprofen, phenylbutazone and aminopyrine as well as flufenamic, meclofenamic and salicylic acids (Table 2). Phenylacetic acid, which is related to indomethacin chemically but which lacks anti-inflammatory activity (Northover, 1964; Durant, Smith, Spickett & Szarvasi, 1965) failed to prevent depolarization (Table 2). However, a number of drugs with well-established anti-inflammatory activity also failed to inhibit heat-induced depolarization. Thus, in the concentrations used, hydrocortisone, chloroquine, benzindamine, isoprenaline and aminophylline were inactive (Table 2).

Other tissues

Venous endothelial cells had membrane potentials similar to those of the aorta, whereas mesothelial cells had a lower potential (Table 3). The hepatic portal vein and the mesentery were both depolarized by the addition of calcium, an effect which was enhanced by histamine or by raising the temperature to 45° C (Table 3). The depolarizing effect of heating to 45° C for 5 h in the presence of calcium 1 mM was reduced by the addition of indomethacin 0.25 mM to the superfusate.

Discussion

In the present experiments endothelial cells from the aorta, the abdominal vena cava and the hepatic portal vein were found to possess similar membrane potentials. Few observations have been made previously of the electrical properties of vascular endothelium. Funaki (1961) recorded membrane potentials from endothelial cells of

Table 2 Effect of anti-inflammatory drugs and related compounds on the membrane potential of aortic endothelial cells during heating to 45°C for 5 h in the presence of 1 mM $CaCl_2$.

Drug	*Concentration (mg/100 ml)*	*Membrane potential (mV negative)** *At 37°C*	*During heating to 45°C. Duration (h)* *1*	*2*	*3*	*4*	*5*
Control	–	37 ± 6	28 ± 5	25 ± 6	21 ± 6	17 ± 5	13 ± 4†
Indomethacin	10	40 ± 5	34 ± 6	31 ± 4	30 ± 4	27 ± 5	25 ± 5‡
Indomethacin	5	38 ± 7	30 ± 5	28 ± 6	27 ± 5	22 ± 4	22 ± 6‡
Indomethacin	2	38 ± 6	29 ± 6	27 ± 4	22 ± 4	19 ± 5	16 ± 5
Cinchocaine	10	39 ± 7	36 ± 6	33 ± 5	30 ± 4	27 ± 4	26 ± 6‡
Cinchocaine	1	38 ± 5	35 ± 5	30 ± 5	29 ± 6	22 ± 6	19 ± 5
Phenylbutazone	40	38 ± 4	35 ± 7	31 ± 4	28 ± 4	25 ± 5	24 ± 3‡
Phenylbutazone	20	35 ± 5	31 ± 6	27 ± 6	24 ± 4	21 ± 4	20 ± 5
Ibuprofen	100	37 ± 6	37 ± 5	32 ± 4	28 ± 3	26 ± 5	26 ± 6‡
Ibuprofen	50	38 ± 5	31 ± 5	23 ± 6	24 ± 7	20 ± 6	18 ± 5
Aminopyrine	20	37 ± 4	36 ± 4	35 ± 5	31 ± 6	28 ± 5	26 ± 5‡
Aminopyrine	5	40 ± 5	29 ± 6	25 ± 4	23 ± 3	17 ± 3	14 ± 4
Flufenamic acid	10	41 ± 6	39 ± 6	33 ± 5	31 ± 6	29 ± 4	27 ± 5‡
Flufenamic acid	2	40 ± 7	30 ± 5	31 ± 6	27 ± 4	21 ± 5	20 ± 4
Meclofenamic acid	10	42 ± 6	40 ± 7	37 ± 5	32 ± 5	29 ± 6	28 ± 5‡
Meclofenamic acid	1	40 ± 7	36 ± 6	34 ± 5	31 ± 5	27 ± 4	25 ± 4‡
Salicylic acid	100	39 ± 6	36 ± 6	30 ± 7	29 ± 4	28 ± 5	25 ± 5‡
Salicylic acid	50	38 ± 5	32 ± 5	30 ± 5	30 ± 3	26 ± 4	24 ± 4
Phenylacetic acid	20	36 ± 5	29 ± 4	27 ± 6	21 ± 5	19 ± 6	14 ± 5
Hydrocortisone	50	39 ± 7	31 ± 6	28 ± 6	23 ± 5	20 ± 5	18 ± 4
Chloroquine	10	36 ± 6	25 ± 6	22 ± 5	20 ± 5	15 ± 4	10 ± 3
Benzindamine	10	38 ± 7	27 ± 7	24 ± 5	20 ± 4	14 ± 5	12 ± 4
Isoprenaline	10	39 ± 8	30 ± 4	28 ± 5	26 ± 3	22 ± 4	20 ± 3
Aminophylline	50	39 ± 6	29 ± 5	28 ± 6	27 ± 3	21 ± 5	19 ± 5

* Mean of at least 30 observations ± s.e. mean.

‡ A significant difference exists between the value marked † and values marked ‡ (Student's *t* test, $P < 0.05$).

Table 3 Membrane potential of endothelial and mesothelial cells from various tissues of the guinea-pig

Tissue	*Temperature (°C)*	*Concentration of $CaCl_2$ (mM)*	*Concentration of histamine (µg/ml)*	*Membrane potential† (mV negative)*
Thoracic aorta	37	0*	0	41 ± 7
Thoracic aorta	37	1	0	37 ± 4
Thoracic aorta	45	1	0	28 ± 5
Thoracic aorta	37	1	100	22 ± 4
Inferior vena cava	37	0	0	39 ± 6
Inferior vena cava	37	1	0	35 ± 5
Inferior vena cava	45	1	0	23 ± 4
Inferior vena cava	37	1	100	22 ± 6
Hepatic portal vein	37	0	0	43 ± 5
Hepatic portal vein	37	1	0	36 ± 7
Hepatic portal vein	45	1	0	25 ± 4
Hepatic portal vein	37	1	100	20 ± 3
Jejunal mesentery	37	0	0	30 ± 4
Jejunal mesentery	37	1	0	28 ± 4
Jejunal mesentery	45	1	0	16 ± 5
Jejunal mesentery	37	1	100	14 ± 3

† After exposure to the stated conditions for 1 hour. The mean of at least 30 observations ± s.e. mean. * Zero signifies that no calcium was added and the superfusate was made up with 0.1 mM EGTA.

small blood vessels in the tongue and foot web of the frog which were a little greater than those found in the present work. Differences between experimental conditions may account in part for the discrepancy. In addition, differences probably exist between the membrane potentials of endothelial cells in blood vessels of different size and location and particularly from different species. Action potentials were not observed by Funaki, or in the present experiments.

Aortic endothelial cells were depolarized by several monovalent metal cations in the present work. The presence or absence of calcium made little difference to the depolarizing effect of potassium. This suggests that, in contrast to the situation in certain other tissues, there is little interaction between these two ions in the endothelial cell membrane. The depolarizing action of potassium has been reported by other workers in a variety of tissues (Adrian, 1956, Dean & Matthews, 1970; Kanno, 1972; Okado, Ogawa, Aoki & Izutsu, 1973). The reduction in membrane potential for a tenfold increase in potassium concentration was 21 mV in the absence of calcium and 20 mV in the presence of calcium 1 mM. These are lower values than have been reported in certain other tissues and indicate that the membrane potential of endothelial cells is dependent upon the trans-membrane concentration gradient of other ions in addition to potassium.

Electrically excitable cells usually depolarize in the absence of extracellular calcium (Frank & Inoue, 1973). Smooth muscle cells appear to retain their membrane potential in the absence of calcium provided that magnesium ions are present (Bülbring & Tomita, 1970). Certain non-excitable cells actually show a hyperpolarization when extracellular calcium is removed (Kanno, 1972). This latter study, however, was conducted in the continued presence of magnesium. In the present experiments it was found that removal of calcium from the bathing fluid did not cause depolarization, whether or not magnesium ions were present. Moreover, the addition of calcium to the bathing fluid in supra-physiological amount actually caused depolarization. Most significantly of all, a concentration of calcium normally present in the blood plasma caused enhancement of the depolarizing effects of histamine and heating to 45° C.

The ability of the trivalent cations of lanthanum, aluminium and iron to antagonize the action of calcium was not totally unexpected since a similar antagonism has been reported for lanthanum by van Breemen, Fabrinas, Casteels, Gerba, Wuytack & Deth (1973). A comparison of the calcium-antagonizing actions of lanthanum, aluminium and iron does not appear to have been made previously. Nevertheless, some of the published pharmacological actions of aluminium are most readily explained in terms of inhibition of the effects of endogenous calcium (Hava & Hurwitz, 1973).

Firm conclusions regarding the possible relationships between the membrane potential of endothelial cells and the leakage of plasma through the endothelium of small blood vessels during inflammation are obviously not yet possible. Four points, however, are worthy of note. Firstly, exposure of mammalian tissues to histamine or to a temperature of 45° C both cause inflammation (Starr & West, 1967). Secondly, the present findings indicate that either histamine or heating to 45° C can depolarize endothelial cells, the effects of both being enhanced by a concentration of calcium existing normally in the blood plasma. Thirdly, indomethacin and members of a group of pharmacologically related substances reduce the binding of calcium to endothelial cell membranes (see Introductory section). Fourthly, these same drugs have been shown in the present experiments to inhibit the ability of calcium to augment the histamine-induced and heat-induced depolarization of endothelial cells. One can only speculate as to how these four separate series of observations are connected. Perhaps, by binding to the endothelial cell membrane, the calcium ions normally present in the blood plasma facilitate inflammatory responses to histamine or heat. In which case, perhaps it is by reducing the binding of calcium to endothelial cell membranes that drugs such as indomethacin exert their anti-inflammatory effect

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INHIBITION OF 5-HYDROXYTRYPTAMINE-INDUCED HUMAN BLOOD PLATELET AGGREGATION BY CHLORPROMAZINE AND ITS METABOLITES

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1 Blood platelets from normal human subjects were isolated and aggregated *in vitro* with adenosine diphosphate (ADP) or 5-hydroxytryptamine (5-HT).

2 The effects of chlorpromazine and 7 major metabolites upon 5-HT-induced aggregation were investigated.

3 All the phenothiazines inhibited 5-HT-induced aggregation when added to platelet rich plasma 3 min prior to 5-HT.

4 There were no qualitative differences in the inhibitory effects, but inhibitory potency varied over a wide range. The decreasing order of potency was monodesmethylchlorpromazine, chlorpromazine, 7-hydroxychlorpromazine, didesmethylchlorpromazine, 3,7-dimethoxychlorpromazine, didesmethylchlorpromazine sulphoxide, chlorpromazine sulphoxide, chlorpromazine nitroxide.

Introduction

The purpose of this investigation was to define a pharmacological action of chlorpromazine (CPZ) which might be used as a technique to measure the pharmacological activity of CPZ and its metabolites in patients during therapy. Chlorpromazine and some of its metabolites are accumulated by human erythrocytes and platelets (Ahtee & Paasonen, 1965; Ahtee, 1966a; Solatunturi & Ahtee, 1968). Mills & Roberts (1967) showed that low concentrations of chlorpromazine (1 nmol/ml) inhibited platelet aggregation *in vitro*, induced by adrenaline and 5-hydroxytryptamine (5-HT). Most important, CPZ inhibited 5-HT-induced platelet aggregation more than the aggregation caused by adenosine diphosphate (ADP). The effective inhibitory concentrations of CPZ were comparable to those found in the plasma of patients treated with CPZ (Curry, 1971).

The clinical effects of prolonged CPZ treatment on human blood platelet aggregation responses have not been investigated. However, it is known that CPZ is extensively metabolized by man, and there are, theoretically at least, 168 possible metabolites (see Usdin, 1971). The poor correlation between CPZ plasma concentrations and the therapeutic responses of schizophrenic patients has led to the proposal that one or more of the chlorpromazine metabolites may be pharmacologically active within the human brain (Curry, 1971; Curry, Lader, Mould & Sakalis, 1972). Recently Sakalis, Chan, Gershon & Park (1973) have found a relationship between plasma concentrations of 7-hydroxylated CPZ metabolites and a good therapeutic response to CPZ treatment in schizophrenics, while a poor response appeared to be associated with the presence of CPZ metabolites with the sulphoxide moiety (CPZSO, NOR_1CPZSO, NOR_2CPZSO, $7OHNOR_1CPZSO$, $7OHNOR_2CPZSO$). We considered that these metabolites might show pharmacological activity by inhibiting platelet aggregation. This paper reports the effects of seven CPZ metabolites upon platelet aggregation responses induced by 5-HT.

Methods

Blood was obtained by venepuncture from normal male and female volunteers aged 18-46 years, who had not taken acetylsalicylic acid for at least 5 days and no other drug for at least 3 days. No females were receiving oral contraceptives. The blood was collected into 0.129 M sodium citrate and centrifuged at 120-150 *g* to prepare citrated platelet rich plasma (PRP) as described by Boullin, Green & Price (1972). Samples (1 ml) of PRP were incubated at 37°C and then platelet aggregation responses were recorded with the platelet aggregometer (Boullin *et al.*, 1972) coupled to an X-Y pen recorder. This type of instrument was found to be particularly satisfactory for measurement of the platelet aggregation rate. We used either a Bryans Model 24000A4, (Bryans Southern Ltd, Mitcham, Surrey), with a chart speed of 6 cm/min giving deflections on the Y axis of 400 μV/cm, or a Tekman TE 200 (Tekman Ltd, Oxford) using a speed of 20 or 40 mm/min giving deflections on the Y axis of 200 μV/cm.

The PRP was stirred at 1000 rev/min at 37°C and the phenothiazines were added 3 min before aggregation was induced by the addition of 5-HT. Responses were quantitated as changes in optical density (light transmittance) expressed in arbitrary units, or as a rate of change (μV/minute)

Drugs

The following drugs were used. 5-hydroxytryptamine creatinine sulphate and adenosine diphosphate (Sigma Chemical Co, St Louis, Mo, USA), chlorpromazine (May & Baker Ltd). CPZ metabolites were obtained from Mr D. Wiles, Littlemore Hospital, Oxford.

We used 7-hydroxychlorpromazine base (7OHCPZ); monodesmethylchlorpromazine maleate (NOR_1CPZ); didesmethylchlorpromazine maleate (NOR_2CPZ); chlorpromazine nitroxide dihydrate (CPZNO); 3,7-dimethoxychlorpromazine hydrochloride (CH_3OCPZ); didesmethylchlorpromazine sulphoxide hydrochloride (NOR_2CPZSO) and chlorpromazine sulphoxide, base (CPZSO). 7OHCPZ and CH_3OCPZ are photolabile (Usdin, 1971) and experiments with these compounds were carried out in a room lit with tungsten filament lamps obscured with yellow filters (Kodak Safelight OB).

All drug concentrations are expressed in terms of molarity of the base.

Results

5-HT induced platelet aggregation, although the effect was small in comparison with aggregation produced by ADP. The effects of 5-HT were approximately 20-30% of the maximal aggregation response produced by ADP, as determined by the change in optical density of PRP. Table 1 shows normal 5-HT aggregation rates, expressed as change in optical density measured on the pen recorder as μV/min, compared with those obtained with equimolar concentrations of ADP in the same subject. This Table shows that with 5-HT concentrations in the range 4-50 nmol/ml, the initial rate of aggregation was maximal and not concentration-dependent for either ADP (Boullin, *et al.*, 1972) or 5-HT. Moreover in no case did the 5-HT aggregation rate exceed 30% of the maximum rate obtained with ADP. The responses to 5-HT were small, transient and consisted of one phase only; irreversible aggregation typically seen with ADP, was seen with 5-HT in only 10% of 40 normal subjects. Also there was considerable individual variation in the aggregation response to 5-HT, maximal change in light transmittance was not obtained with any predictable 5-HT concentration and in some cases inhibition was observed with concentrations of 50 nmol/ml. These data confirm earlier work (O'Brien, 1964; Mills & Roberts, 1967; Baumgartner & Born, 1968). Accordingly we tested the responses of each subject with a range of 5-HT concentrations from 4.0 to 50 nmol/ml. Having established the 5-HT concentration producing the maximal aggregation effect, we then investigated the inhibitory actions of the phenothiazine compounds on the aggregation produced by this concentration.

Inhibition of 5-hydroxytryptamine aggregation by chlorpromazine and metabolites

Figure 1 compares the effects of 0.1, 1.0, 10.0 and 100 nmol/ml concentrations of CPZ, 7OHCPZ and CPZSO and illustrates the differing inhibitory potencies of CPZ and metabolites upon 5-HT-induced aggregation. The figure also shows that these phenothiazines did not produce qualitative differences in the 5-HT aggregation responses. That is, aggregation was invariably transient and reversible. Similar results were obtained with the 5 other chlorpromazine metabolites, although there were differences in their inhibitory potency. When these inhibitory effects were expressed in terms of percentage inhibition of the maximum rate of

Table 1 Maximum rates of platelet aggregation produced by 5-hydroxytryptamine (5-HT)

Dose of drug added (nmol/ml)	*5-HT*	*ADP*	*5-HT response as % of ADP response*
	(Rate of change of optical density)		
4	1220	4114	29.7
n = 7	±271	±242	±4.7
20	1057	4490	23.5
n = 11	±242	±865	±4.3
50	1400	4207	33 3
n = 5	±278	±403	±5.5

Samples (1 ml) of PRP were aggregated with 5-HT or ADP at 37°C as described in the methods section. Maximal rates of aggregation (μV/min) were calculated from the initial rate of aggregation measured during 15 s after addition of drug. Values are the mean with s.e. mean.

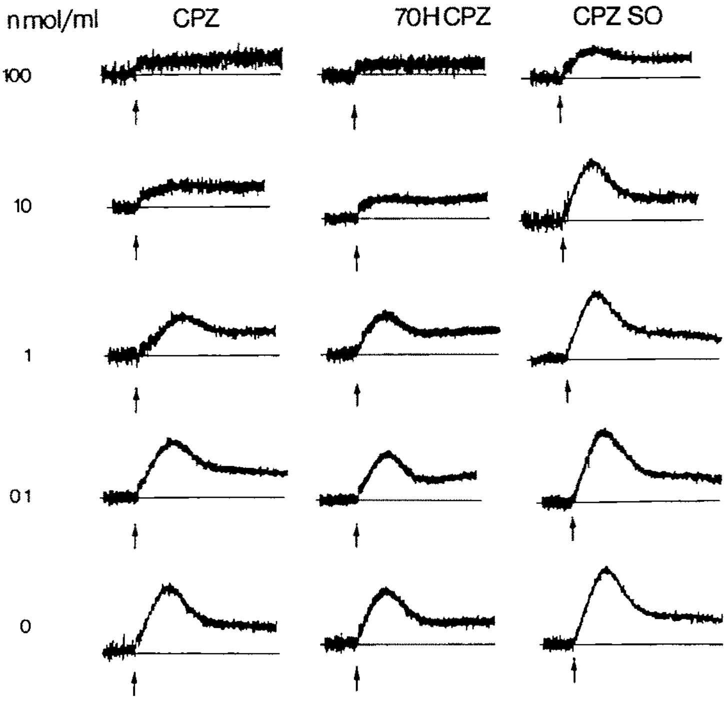

Figure 1 Inhibitory effects of various concentrations of chlorpromazine (CPZ), 7 hydroxy-CPZ (7OH CPZ) and CPZ sulphoxide (CPZSO) compared to control responses. CPZ compounds were added to PRP 3 min before induction of aggregation with 5-HT 20 μM (at the arrows).

aggregation produced by a standard dose of 5-HT, we obtained the data shown in Figure 2. This compares the inhibitory effects of the 7 metabolites with CPZ. Table 2, column I, shows the differences in potency calculated on the basis of the ID_{50}, the phenothiazine concentration required to produce 50% inhibition of the maximum 5-HT aggregation rate. These results indicate that there is a wide spectrum of inhibitory activity of the chlorpromazine metabolites, but all show some degree of pharmacological activity on platelet aggregation *in vitro*.

Table 2 Comparative activities of chlorpromazine (CPZ) metabolites in blocking amine sensitive cellular systems

Metabolites	*I* *5-HT induced platelet aggregation*	*II* *Dopamine-sensitive adenylate cyclase*
	(ID_{50} nmol/ml)	
NOR$_1$CPZ	0.9	6.0
CPZ	2.0	1.3
7OHCPZ	3.1	14.0
NOR$_2$CPZ	4.0	11.0
3,7,CH$_3$OCPZ	19.0	Not tested
NOR$_2$CPZSO	50.0	Not tested
CPZSO	68.0	> 100
CPZNO	120.0	60.0

Data in column I are from the present work; data in column II are from Miller & Iversen (1974).

Discussion

It has been established that chlorpromazine is metabolized to a large number of derivatives in man, and that some of these substances possess potent pharmacological activity (for review see Usdin, 1971).

In general, the pharmacological activity of CPZ metabolites in blocking 5-HT-induced platelet aggregation parallels their activity in other systems, particularly in the central nervous system. Posner, Hearst, Taylor & Cosmides (1962), Manian, Efron & Goldberg (1965) found that NOR$_1$CPZ and 7OHCPZ were the most active metabolites in a series of tests which measured animal behaviour. We found that the demethylated derivative, NOR$_1$CPZ, is more potent in inhibiting platelet aggregation than CPZ itself; that NOR$_2$CPZ and 7OHCPZ are somewhat less potent,

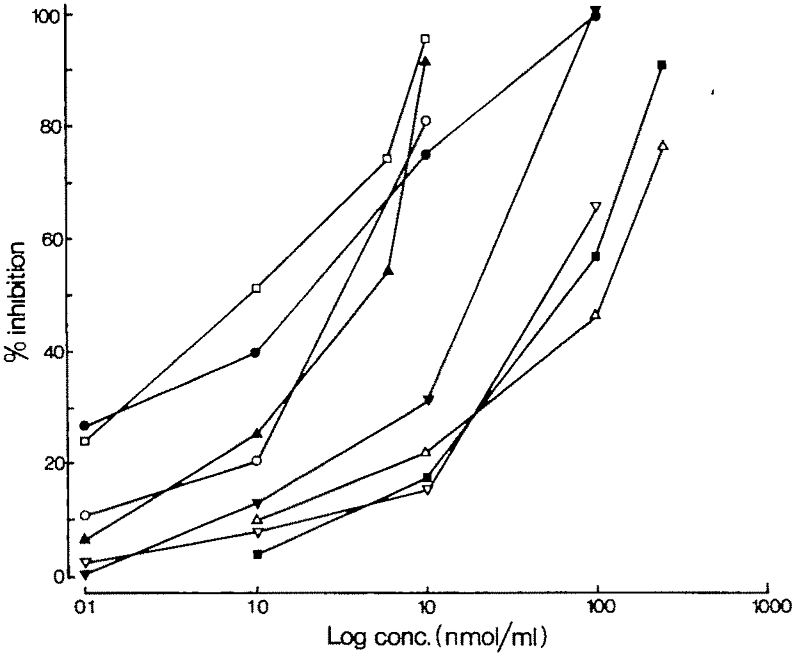

Figure 2 Comparative potency of chlorpromazine (CPZ) and 7 major metabolites in blocking 5-hydroxytryptamine-induced aggregation. Blockade of aggregation was assessed as described in methods section. Each point represents the mean value of 7 to 19 experiments with the standard error not exceeding 10% of the mean value. (●) CPZ; (○) 7OH; (■) CPZSO; (□) NOR 1; (▲) NOR 2; (▽) CPZ NO; (△) 3,7,MeO; (▼) NOR 2 SO.

while the N-oxide and sulphoxide metabolites have less than 1/10 the potency of CPZ. Some of these metabolites exhibit a remarkably similar order of potency to that seen with the dopamine-sensitive adenylate cyclase of rat striatum described by Miller & Iversen (1974) (Table 2, column II). Thus there is a precedent for the fact that CPZ metabolites have some pharmacological activity in the broadest sense in the brain and blood platelets. Differences in binding to platelet cells and plasma proteins may also influence pharmacological action of CPZ and metabolites. The Finnish group in Helsinki made an extensive investigation of the physicochemical properties of CPZ metabolites; their haemolytic effects on red cells, binding to platelets, and release of catecholamines from adrenal glands. CPZSO was bound less to serum proteins than CPZ or NOR_1CPZ (Ahtee, Mattila & Vapaatalo, 1967) and smaller amounts were taken up by platelets (Ahtee & Paasonen, 1966). Subcellular fractionation showed that most CPZSO was in the supernatant platelet fraction, whereas CPZ and NOR_1CPZ were particulate bound (Solatunturi & Ahtee, 1968). The pharmacological activity of these 3 phenothiazines paralleled their platelet binding characteristics (Ahtee & Paasonen, 1965; Ahtee, 1966a), and their ability to release catecholamines from adrenal glands (Vapaatalo, Ahtee & Paasonen, 1966).

Comparison of the surface active properties of CPZ with NOR_1CPZ and CPZSO (Ahtee, 1966b) showed that CPZSO had the lowest potency. On the basis of this work, differences in pharmacological potency might be attributed to the differences in surface activity and protein binding. However the concentrations required to produce these effects were at least 10^{-4} M. Such concentrations of CPZ release 5-HT from platelets (Bartholini, Pletscher & Gey, 1964) and cause gross ultrastructural changes, including disruption of the plasma membrane with consequent leakage of the intracellular contents (Telkkä, Nyholm & Paasonen, 1964).

On the other hand, the pharmacological effects reported here and the effects described by Miller & Iversen (1974) upon brain adenylate cyclase were usually obtained with concentrations in the range 10^{-5} M to 10^{-8} M. In patients treated with CPZ we found that plasma concentrations of CPZ, NOR_1CPZ and CPZSO range between 30-400 pmol/ml. According to Fig. 2, CPZ and

NOR_1CPZ are both pharmacologically active in this concentration range.

We would like to thank the following for gifts of drugs: May & Baker Ltd (chlorpromazine), Mr D. Wiles, Littlemore Hospital, Oxford (CPZ metabolites; these were originally obtained from Dr A.A. Manian, Psychopharmacology Research Branch, National Institute of Mental Health, HSMHA, Rockville, Md 20852, U.S.A.).

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ACTIVATION OF HISTIDINE DECARBOXYLASE BY H_2-RECEPTOR BLOCKADE: MECHANISM OF ACTION

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1 Treatment with histamine H_2-receptor antagonists, which inhibit basal acid secretion, was found to activate rat stomach histidine decarboxylase. At the same time the serum gastrin concentration was greatly increased.

2 In antrectomized rats neither the enzyme activity nor the serum gastrin concentration was affected by the treatment.

3 In analogy with previous observations on other inhibitors of acid secretion we suggest that the H_2-receptor antagonists stimulate gastrin release through their effect on acid secretion and that the raised serum gastrin level is responsible for the enzyme activation.

Introduction

A histamine-forming enzyme, histidine decarboxylase, is found in the rat stomach. Recently, Maudsley, Kobayashi, Williamson & Bovaird (1973) reported that treatment with H_2-receptor antagonists, burimamide or metiamide, activates this enzyme.

In previous studies evidence was obtained that the activity of rat stomach histidine decarboxylase varies with the serum gastrin level (Håkanson, Kroesen, Liedberg, Oscarson, Rehfeld & Stadil, 1974). Furthermore, the enzyme-activating effect of various surgical and pharmacological treatments was abolished by antrectomy whereas that of gastrin or gastrin analogues was not (Johnson, Jones, Aures & Håkanson, 1969, Aures, Johnson & Way, 1970; Håkanson & Liedberg, 1970, 1971a, b; 1972; Håkanson, Liedberg & Sjölund, 1972; Håkanson, Liedberg & Oscarson, 1973a; Håkanson, Liedberg, Oscarson, Rehfeld & Stadil, 1973b). This study is concerned with the role of endogenous gastrin in the enzyme activation after H_2-receptor blockade.

Methods

Adult male albino rats (Wistar strain, 150-200 g body weight) were used. Antrectomy was performed on eight rats by resection of the distal half of the glandular stomach (the pyloric gland area together with the adjacent portion of the oxyntic gland area) and the duodenal bulb. Gastrointestinal continuity was re-established by gastroduodenostomy end-to-end (for details see Håkanson & Liedberg, 1970, 1972). Operated rats were allowed to recover for at least three weeks before they were used in experiments.

Burimamide or metiamide (Smith, Kline and French) were dissolved in 0.1 M sodium acetate-acetic acid buffer, pH 5.0, at 70-80°C to a concentration of 10 mg/ml. After cooling at room temperature the drugs were injected intraperitoneally in doses of 100 mg/kg three times at 2 h intervals. Controls received acetate buffer. The rats were fasted for 48 h (free access to water) before experiments, and they were killed 2 h after the last injection. Blood was drawn (until exsanguination) from the aorta under diethyl ether anaesthesia.

The serum was analysed for gastrin by radioimmunoassay (Stadil & Rehfeld, 1971, 1973). The antiserum (No. 2604; Rehfeld, Stadil & Rubin, 1972) employed in the present study was used in a final dilution of 1 : 200 000. It binds four gastrin components of different molecular size (Rehfeld, Stadil & Vikelsøe, 1974). Monoiodinated [^{125}I]-synthetic human gastrin I was employed as tracer (Stadil & Rehfeld, 1972). The only known naturally occurring peptide which cross-reacts in the assay is cholecystokinin, the ratio in molar terms between ID_{50} for synthetic human gastrin I and pure porcine cholecystokinin is 0.002. The accuracy of the assay for the determination of rat serum gastrin has been evaluated previously (Håkanson *et al.*, 1974). The gastrin concentration is expressed as pg equivalent of synthetic human gastrin I per ml.

The stomachs were taken out, cut open along the major curvature and washed with ice-cold 0.9% w/v NaCl solution (saline). The mucosa was scraped off the oxyntic gland area and homogenized in 0.1 M phosphate buffer, pH 7.0 (final tissue concentration 100 mg wet weight per ml). Enzyme activity was measured as $^{14}CO_2$ produced from [1-^{14}C]-L-histidine. The reaction mixture (0.5 ml) contained 0.4 ml homogenate, 10^{-5} M pyridoxal-5-phosphate, 5 x 10^{-4} M glutathione and 4 x 10^{-4} M [1-^{14}C]-L-histidine (1.3 mCi/mM) added in this order. The mixture was gassed with nitrogen for 5 min at 0°C. After 1 h at 37°C the reaction was terminated by the addition of 0.5 ml 10% trichloroacetic acid, and the evolved $^{14}CO_2$ was trapped during 30 min at 37°C on a filter paper strip, previously immersed in Protosol and placed in a centre well in the reaction vessel. The radioactivity of the filter paper strip was then determined in a liquid scintillation spectrometer. The results were corrected for non-enzymatic decarboxylation by incubating identical samples with [1-^{14}C]-D-histidine instead of [1-^{14}C]-L-histidine. Blanks never exceeded 190 ct/minute. Duplicate assays were run in all experiments. The reproducibility of the assay was such that the standard deviation of 15 identical samples (aliquots of a pooled extract of gastric mucosa from freely fed rats) was 6.6%. Enzyme activities are expressed as pmol CO_2 produced per mg tissue (wet weight) and hour. For further details of the procedure see Håkanson (1970) and Håkanson *et al.* (1974).

Gastric secretion was studied in 300-350 g rats fitted with chronic gastric cannulae, by a slight modification of the technique described by Bel, Levrat, Nesmos & Girard (1966). Before experiments the fistula rats were deprived of food but not of water for 24 hours. The fistula rats were immobilized without anaesthesia in Bollman type cages, the cannulae opened and the stomachs rinsed with warm saline. Saline (10 ml) was given subcutaneously to replace fluid loss during the experiment. The cannulae were allowed to drain

Table 1 Effect of H_2-receptor blockade on serum gastrin concentration and gastric histidine decarboxylase activity

	Treatment	*Serum gastrin (pg SHG Eq/ml)*	*Histidine decarboxylase activity (pmol CO_2 mg^{-1} h^{-1})*
		Mean ± s.e. mean (n)	
Unoperated rats	Controls	46 ± 4 (30)	3.7 ± 0.3 (23)
	Burimamide (100 mg/kg)	118** ± 21 (15)	15.8*** ± 3.0 (10)
	Metiamide (100 mg/kg)	223*** ± 48 (14)	33.0*** ± 4.0 (14)
Antrectomized rats	Controls	35 ± 7 (4)	3.1 ± 0.4 (4)
	Metiamide (100 mg/kg)	41 ± 3 (4)	3.5 ± 1.0 (0.4)

Determinations of serum gastrin (in terms of synthetic human gastrin I (SHG)) and histidine carboxylase activity were usually made on the same animal. All rats received three intraperitoneal injections of burimamide or metiamide 2 h apart and were killed 2 h after the last injection.

Significant differences between controls and drug-treated animals are marked by ** for $0.001 < P < 0.01$ and *** for $P < 0.001$ (Student's *t* test).

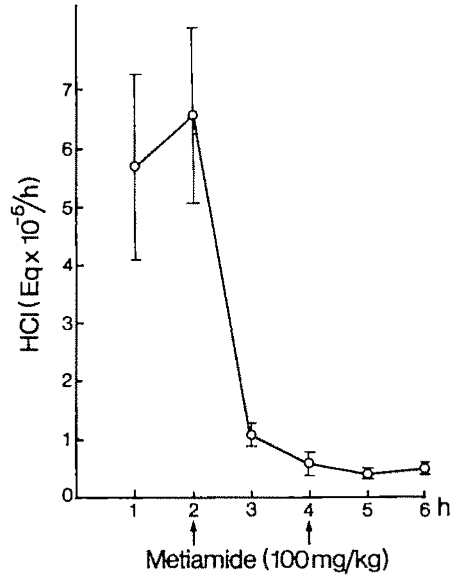

Figure 1 Inhibitory effect of metiamide on basal acid secretion in seven gastric fistula rats. Mean values are given, vertical bars indicate s.e. mean.

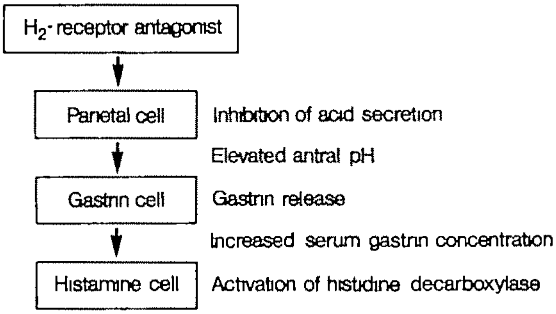
H$_2$-receptor antagonist
Parietal cell
Inhibition of acid secretion
Elevated antral pH
Gastrin cell
Gastrin release
Increased serum gastrin concentration
Histamine cell
Activation of histidine decarboxylase

Figure 2 Proposed chain of events leading to activation of histidine decarboxylase in the rat stomach after H$_2$-receptor blockade. The H$_2$-receptor antagonists act on the parietal cells to inhibit acid secretion. As a consequence, pH in the antral lumen is raised. This stimulates the gastrin cells to release gastrin. The increased serum gastrin concentration causes activation of gastric histidine decarboxylase.

freely for 1 h, and two 1 h samples of basal secretion were collected. Metiamide (100 mg/kg) was given intraperitoneally after which two 1 h samples were collected, the injections were repeated and two more 1 h samples were collected. The volumes were measured and the hourly acid output was determined by titration with 0.02 N NaOH, using phenolphthalein as indicator. Acid output is expressed as mEq HCl per hour.

Results

In confirmation of previous reports we found that H$_2$-receptor antagonists inhibited basal acid secretion (Figure 1) (cf. Black, Duncan, Durant, Ganellin & Parsons, 1972; Black, Duncan, Emmett, Ganellin, Hesselbo, Parsons & Wyllie, 1973) and activated gastric histidine decarboxylase (Table 1) (cf. Maudsley *et al.*, 1973). We also found that the gastrin concentration in serum was greatly increased (Table 1). In antrectomized rats, the H$_2$-receptor antagonists failed to activate the enzyme and the serum gastrin concentrations were not raised above the basal level (Table 1).

Discussion

The present results show that H$_2$-receptor antagonists raise the serum gastrin level as well as the histidine decarboxylase activity. This is in agreement with the hypothesis that the activity of this enzyme is regulated by the serum gastrin concentration. The great majority of gastrin cells in the digestive tract of the rat are found in the antrum and in the regions immediately adjacent to the antrum (Larsson, Håkanson, Rehfeld, Stadil & Sundler, 1974). However, the serum gastrin concentration was only lowered to a minor extent by antrectomy, possibly because there exists an as yet unrecognized source of gastrin immunoreactivity outside the digestive tract. The H$_2$-receptor antagonist metiamide failed to raise the serum gastrin level in antrectomized rats. The histidine decarboxylase activity of these animals was also unaffected by metiamide. This observation lends further support to the view that gastrin is a mediator of the enzyme activation.

The inhibition of basal acid secretion produced by injection of metiamide may possibly explain why H$_2$-receptor antagonists increase the serum gastrin level (Figure 2). Conceivably, the elevation of antral pH that results from the inhibition of acid secretion facilitates gastrin release (Becker, Reeder & Thompson, 1973). The H$_2$-receptor antagonists may thus be added to the list of agents that non-specifically activate rat stomach histidine decarboxylase through an inhibitory effect on acid secretion. This list includes 'anti-gastrin' (SC-15396) (Håkanson & Liedberg, 1971b), prostaglandin E$_1$ (Håkanson, *et al.*, 1973a), deglycyrrhizinized liquorice (Håkanson *et al.*, 1973b), atropine and hexamethonium (Håkanson *et al.*, 1974).

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AN ANALYSIS OF THE PHENOMENON OF ACUTE TOLERANCE TO MORPHINE IN THE GUINEA-PIG ISOLATED ILEUM

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1 The observations which Paton (1957) interpreted as 'acute tolerance' and 'dependence' have been confirmed for coaxially stimulated segments of guinea-pig ileum and extended to the contractions evoked by field stimulation in the myenteric plexus-longitudinal muscle preparation. Evidence is adduced that the morphine receptors of the myenteric plexus are not involved in the two phenomena.

2 The contraction of the longitudinal muscle depressed by low concentrations of morphine, or levorphanol, can be restored to control level not only by high concentrations of morphine but also by levorphanol and equally well by its (+)-isomer, dextrorphan, which does not fulfil the stereospecific requirements of the morphine receptor. Acetylcholine output was not increased.

3 When, after restoration of the twitch by high concentrations of morphine, the drug is washed out, contractions become depressed. This effect cannot be due to 'dependence' because either morphine or its antagonist, naloxone, restore the twitch again.

4 In the concentrations used, morphine, levorphanol and dextrorphan inhibit the cholinesterase of homogenates of the myenteric plexus-longitudinal muscle preparation by 10-15%, Since a concentration of physostigmine which causes a similar inhibition also restores the twitch, it is concluded that the described phenomena are best explained by the anti-cholinesterase effects of the drugs.

Introduction

When Paton (1957) showed that morphine-like drugs depressed the evoked contraction of the longitudinal muscle of the guinea-pig isolated ileum, he also described two phenomena which he interpreted as 'acute tolerance' and 'dependence'. A depression of contraction was first obtained with a relatively low concentration of morphine (2.6 μM); increases in the concentration to 5.3 and 58 μM did not lead to any further depression of the twitch. When the morphine was then washed out, there was a decrease in the twitch height which could, however, be restored to the control or pre-morphine level by successive additions of morphine (up to 530 μM) to the bath fluid. Paton proposed that the decrease in sensitivity to morphine after exposure to a relatively low concentration may be likened to 'tolerance' and the depression of the twitch after washing out of the high concentration and its restoration by a similar or higher concentration may be an expression of 'dependence'. Similar findings were obtained with nalorphine. These observations have been confirmed in our and other laboratories (Fennessy, Heimans & Rand, 1969).

The concentrations of morphine that counteract the depression of lower concentrations, are very high indeed when compared with the concentration of 0.07 μM that is required to depress the twitch by 50% (Kosterlitz & Watt, 1968). Since Gyang & Kosterlitz (1966) have shown that morphine has also weak antagonist activity, the possibility has to be considered that the high concentrations of morphine antagonize the depressant effects of the low concentrations. When Paton made his original observations, the only available morphine-antagonists were nalorphine and levallorphan, both of which have also considerable agonist activity. With the introduction of naloxone, which is a morphine antagonist without significant agonist activity, it has become possible to test whether or not the proposed interpretation is correct. A preliminary report of the results was given to a joint meeting of the British and German

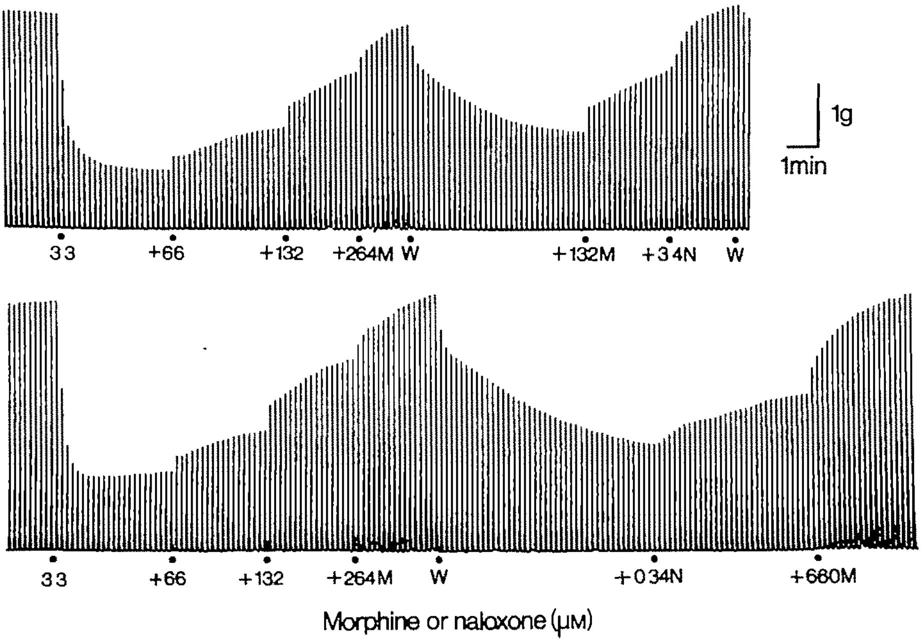

Figure 1 The effects of low and high concentrations of morphine and naloxone on the evoked contractions of the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum. Field stimulation (0.1 Hz, 0.5 ms, supramaximal voltage) and isometric recording. The figures under the tracing indicate concentrations (μM) of morphine (M) or naloxone (N). W denotes washing out of drugs.

Pharmacological Societies (Waterfield & Kosterlitz, 1973).

Methods

Experimental procedure

All experiments were performed on the guinea-pig isolated ileum; the terminal section was used after the 10 cm nearest to the ileo-caecal junction had been discarded. The techniques used were those described earlier (Kosterlitz, Lydon & Watt, 1970). The bath fluid was a modified Krebs solution of the following composition (mM): NaCl 118, KCl 4.75, $CaCl_2$ 2.54, KH_2PO_4 1.19, $MgSO_4$ 1.20, $NaHCO_3$ 25, glucose 11; it also contained mepyramine maleate (0.125 μM) and, for the experiments on the myenteric plexus-longitudinal muscle preparation, choline chloride (20 μM). It was bubbled with 95% O_2 and 5% CO_2. The volume of the bath fluid was 40 ml when contractions of a segment of ileum were recorded, and 3 ml in all other experiments. The temperature was 36°C. The stimuli were 1.3 times maximal rectangular pulses of 0.5 ms duration at a frequency of 0.1 Hz; the whole segment of ileum was stimulated coaxially and the myenteric plexus-longitudinal muscle preparation by field stimulation. The twitch-like contractions of the longitudinal muscle were recorded isometrically by a mechano-electrical transducer and displayed on a pen oscillograph or a potentiometric recorder.

The acetylcholine released from the electrically stimulated myenteric plexus-longitudinal muscle preparation in the presence of physostigmine sulphate (7.7 μM) was assayed on the guinea-pig ileum (Kosterlitz *et al.*, 1970).

For the estimation of total cholinesterase, weighed strips of longitudinal muscle with attached myenteric plexus (about 350 mg) were ground by hand in a ground glass homogenizer containing 7 ml of ice-cold incubation medium (NaCl 130 mM, $MgCl_2$ 35 mM, $NaHCO_3$ 31 mM, gassed with 5% CO_2 and 95% N_2; pH 7.4). Cholinesterase was determined in a Gilson respirometer, at 37°C, with acetylcholine perchlorate (14 mM) as substrate. The mean cholinesterase activity of homogenized tissue was 148.5 ± 5.3 μl

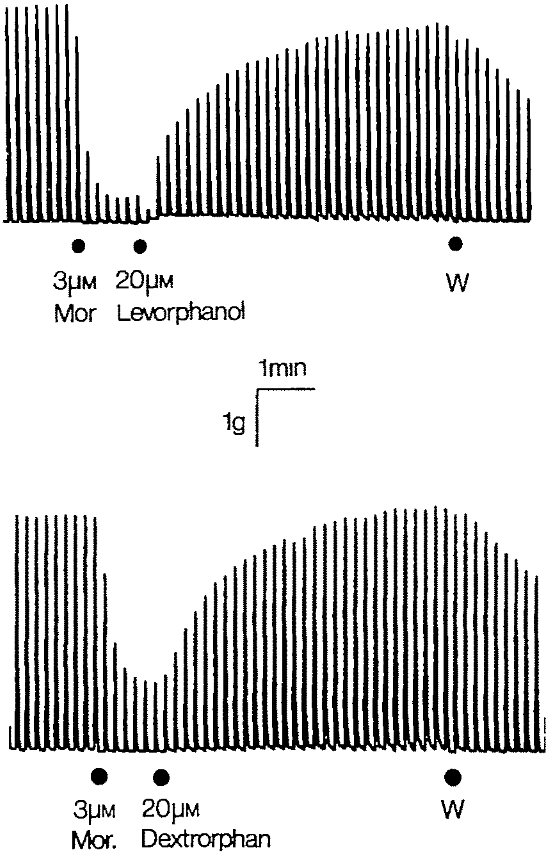

Figure 2 The effects of high concentrations (20 μM) of levorphanol and dextrorphan on the height of the contractions of a myenteric plexus-longitudinal muscle preparation depressed by morphine (Mor.). Stimulation and recording as in Figure 1. W, washing out of drugs.

CO_2 min^{-1} g^{-1} wet weight. Drugs were added 5 min before the start of measurements which were continued for 1 hour.

Drugs

Drugs used were: acetylcholine chloride, choline chloride, physostigmine sulphate, hexamethonium bromide, mepyramine maleate, histamine acid phosphate, bradykinin, morphine hydrochloride, nalorphine hydrobromide, levorphanol tartrate, dextrorphan tartrate, naloxone hydrochloride, cyclazocine ({±}α-2-cyclopropylmethyl-5,9-dimethyl-2′-hydroxy-6,7-benzomorphan).

Stock solutions were made up in distilled water, cyclazocine was dissolved after addition of the required amount of HCl. Concentrations are given as μM or nM with respect to base.

Results

Paton (1957) obtained his original findings on a segment of ileum. We confirmed these observations on the myenteric plexus-longitudinal muscle preparation (Figure 1). A concentration of 3.3 μM of morphine depressed the twitch by 72%. When the morphine concentration was raised to about 70 μM, the twitch height increased; at 200 μM morphine, the depression was reduced to 26.5% and at 464 μM it was only 4%. On washing out morphine, the twitch height decreased immediately and reached a minimum after 5 to 6 min, a phenomenon likened by Paton to 'dependence'. If this interpretation is correct, additions of morphine and of the antagonist naloxone should have opposite effects, morphine should increase the twitch height and naloxone decrease it by evoking an 'abstinence syndrome'. This expectation, however, was not fulfilled, since both drugs caused an increase in twitch height. The order of applying morphine and naloxone did not affect the results (Figure 1).

Similar results were obtained when the initial depression of the longitudinal contraction of a segment of ileum was caused by compounds which had not only agonist but also antagonist activity. Thus, the depressant effects of nalorphine (0.48 μM) or of cyclazocine (0.009 μM) were reversed by morphine (660 μM).

If the increase in twitch height caused by high concentrations of morphine were due to interaction with morphine receptors, this effect would be expected to be stereospecific. Therefore, the actions of the (−)-isomer of 3-hydroxy-*N*-methylmorphinan, levorphanol and of its (+)-isomer, dextrorphan, were investigated. It was found that both isomers were effective in increasing the twitch height depressed by 3 μM morphine (Figure 2).

The similarity of the effects of morphine and naloxone and the lack of stereospecificity suggest that the restoring effects of high concentrations of morphine are not due to an action on morphine receptors which, on activation, decrease acetylcholine release from the myenteric plexus of the guinea-pig ileum. This view was tested by determining the output of acetylcholine evoked by electrical stimulation before and after addition of low and high concentrations of morphine, levorphanol, dextrorphan and naloxone.

As has been shown previously for segments of

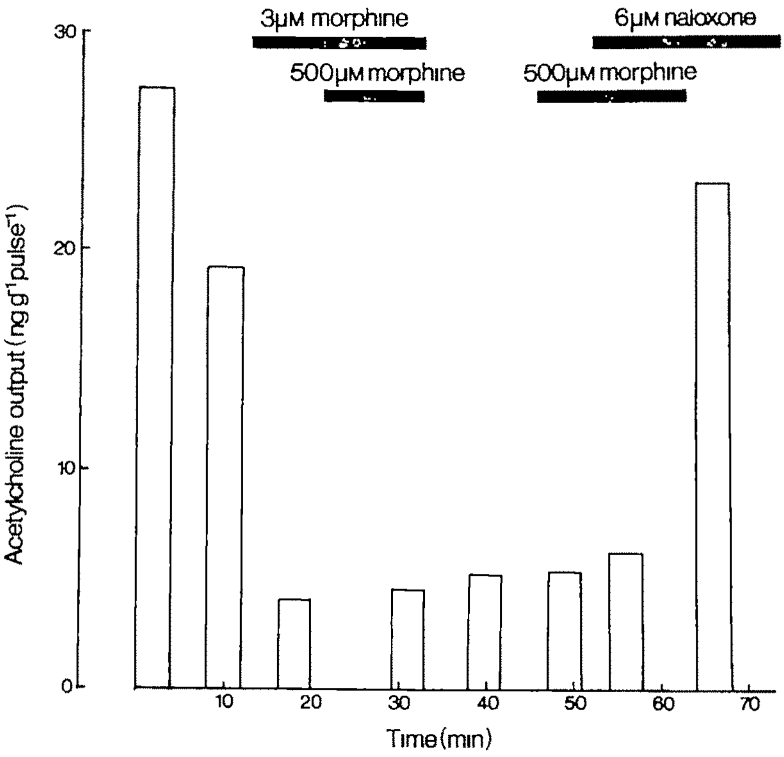

Figure 3 The effects of low and high concentrations of morphine and naloxone on the acetylcholine output of the myenteric plexus-longitudinal muscle preparation in the presence of physostigmine (7.7 μM). Stimulation as in Figure 1. Abscissae, time (min); ordinates, acetylcholine output (ng g^{-1} $pulse^{-1}$). The horizontal bars indicate presence of drugs.

ileum (Paton, 1957) and for the myenteric plexus-longitudinal muscle preparation (Cowie, Kosterlitz & Watt, 1968; Paton & Zar, 1968), a low concentration of morphine (3 μM) reduced acetylcholine output evoked by electrical field stimulation (Figure 3). When the concentration of morphine was raised to 500 μM, there was no restoration of acetylcholine output; after washing out this high concentration of morphine, acetylcholine output did not change for up to 5 minutes. Thus, when the twitch height was increased by raising the morphine concentration or when it was decreased immediately after washing out morphine (Figure 1a, b), there was no change in acetylcholine output. When a relatively small amount of naloxone (6 μM) was added in presence of the high concentration of morphine, there was, in this particular experiment, no increase in acetylcholine output which, however, returned to the control level when morphine was washed out while naloxone remained in the bath fluid. In a series of 5 experiments, morphine (3 μM) lowered the control acetylcholine output (ng g^{-1} $pulse^{-1}$) from 20.5 to 6.6 ($P < 0.01$). When the morphine concentration was raised to 500 μM, the acetylcholine output was 6.5; addition of naloxone (6 μM) raised the output to 8.5 ($P < 0.025$). When morphine was washed out while naloxone was still present, the output rose to 20.2 ($P < 0.01$).

The effects of levorphanol and dextrorphan on evoked contractions of the longitudinal muscle and on acetylcholine output are shown in Figure 4. The results were obtained in two separate series; in the experiments on the contraction, no physostigmine was added to the bath fluid while for the determination of acetylcholine output physostigmine had to be used. A low concentration of levorphanol (0.4 μM) decreased the contraction and lowered acetylcholine output. When levorphanol or dextrorphan was then added to give a high concentration (70 μM), the size of the contraction became larger than that obtained

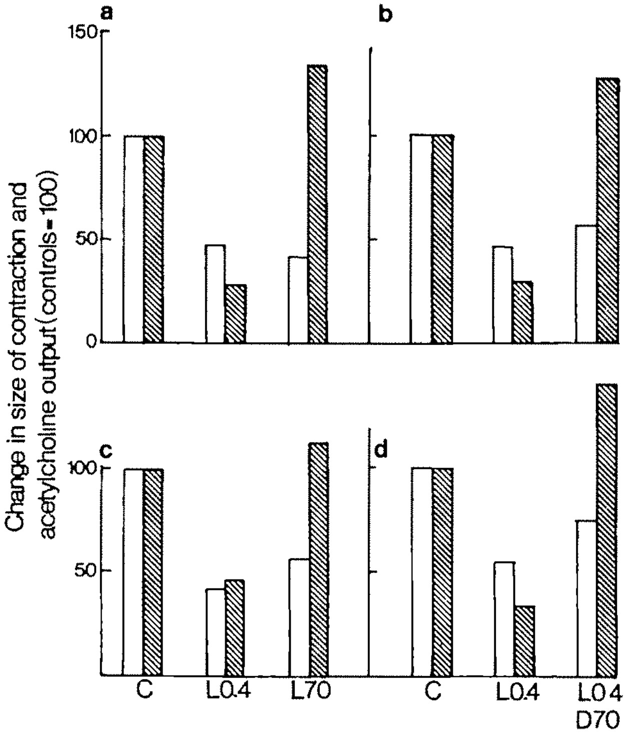

Figure 4 Myenteric plexus-longitudinal muscle preparation. The effects of low and high concentrations of levorphanol and dextrorphan on the size of contractions in the absence of physostigmine and the evoked acetylcholine output measured in separate preparations in the presence of physostigmine. Stimulation and recording as in Figure 1. Abscissae, C, controls; L 0.4, 0.4 μM levorphanol; L 70, 70 μM levorphanol; D 70, 70 μM dextrorphan. Ordinates, changes in acetylcholine output (clear columns) or contractions (shaded columns) (control values = 100) (a and b) control output 23 ng g^{-1} $pulse^{-1}$; (c and d) hexamethonium (140 μM) present throughout, control outputs, 19 (c) and 13 (d) ng g^{-1} $pulse^{-1}$.

before any drug had been added although the output of acetylcholine was unaltered or raised only very little. Addition of hexamethonium which lowered the control output of acetylcholine, did not alter the described effects of levorphanol or dextrorphan on either contraction or acetylcholine output.

Since the findings so far reported indicate that in this preparation the phenomena likened by Paton (1957) to tolerance and dependence are not due to prejunctional effects, the possibility of changes in the sensitivity of the smooth muscle cells to acetylcholine was examined. The dose-response curve for acetylcholine was reversibly shifted to the left by morphine (500 μM) and levorphanol (70 μM) but there was no increase in the sensitivity to histamine or bradykinin in the presence of hyoscine (Figure 5). Similar results were obtained with dextrorphan (70 μM).

These observations suggest that the increase in the size of contraction caused by high concentrations of morphine, levorphanol or dextrorphan is possibly due to a partial inhibition of cholinesterase present in the preparation particularly since it is known that these compounds inhibit acetylcholinesterase in brain homogenates (e.g. Harris & Dewey, 1972) and that acetylcholinesterase is present in the myenteric plexus and butyrylcholinesterase in the longitudinal muscle (Ambache, Freeman & Hobbiger, 1968). The inhibitory effects of physostigmine, levorphanol, dextrorphan and morphine on total cholinesterase are shown in Figure 6. The following ED_{50} values (μM) were obtained from calculated regression equations: physostigmine 0.96, levorphanol 59, dextrorphan 141 and morphine 3500. Although these values are higher than those found by Harris & Dewey (1972), who used rat

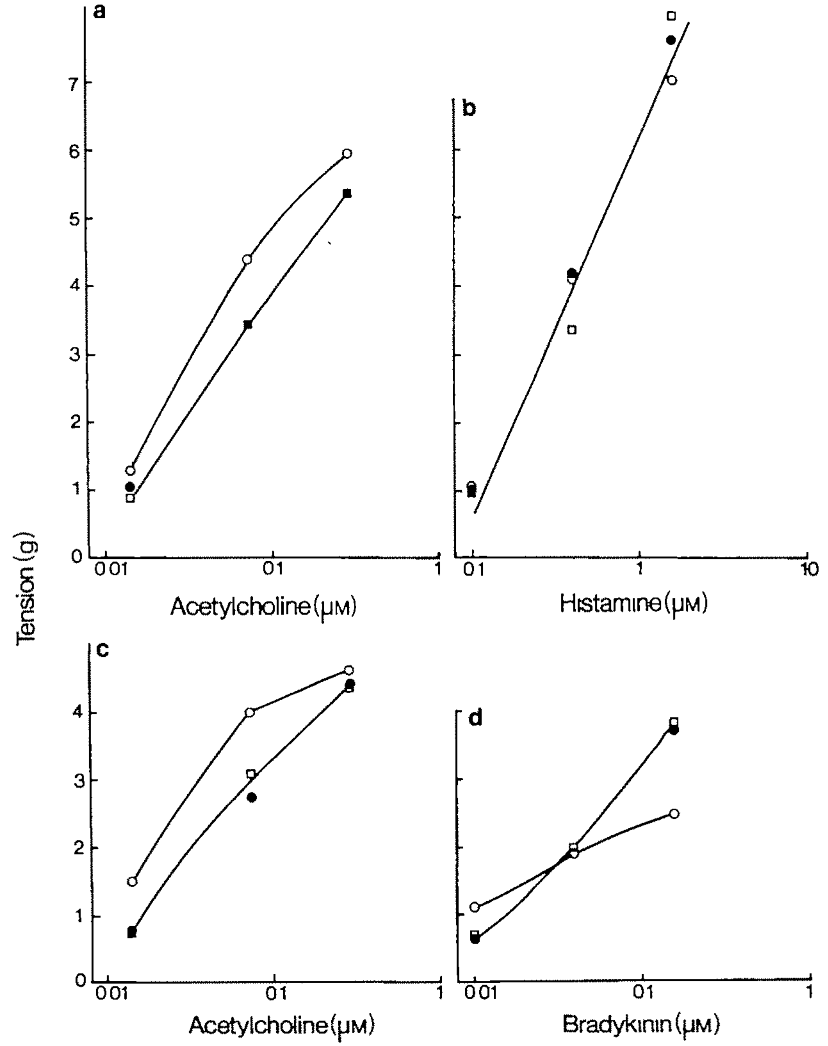

Figure 5 The effects of morphine (a and b, 500 μM) and levorphanol (c and d, 70 μM) on the size of contractions produced by acetylcholine (a and c), and, in the presence of hyoscine 0.76 μM, histamine (b) and bradykinin (d). Isometric recording. Abscissae, concentration (μM); ordinates, tension (g). Controls (●); in presence of drug (○), after washing out (□).

brain homogenates as the source of enzyme and thiocholine as substrate, the rank order is the same as in their experiments.

Finally, it was found that 0.1 μM physostigmine, which inhibited cholinesterase to the same extent as 500 μM morphine, also restored the twitch depressed by morphine 3 μM.

Discussion

The observations which Paton (1957) interpreted as 'acute tolerance' and 'dependence' have been confirmed for segments of the guinea-pig ileum and similar findings have now been obtained on the myenteric plexus-longitudinal muscle preparation. The analysis of these observations has shown, however, that the morphine receptors present in the myenteric plexus are not responsible for these phenomena.

First, the (+)-isomer, dextrorphan which is not a narcotic analgesic, is as effective as the (−)-isomer, levorphanol, in restoring the contraction depressed by morphine. Secondly, morphine and the antagonist naloxone should have opposite

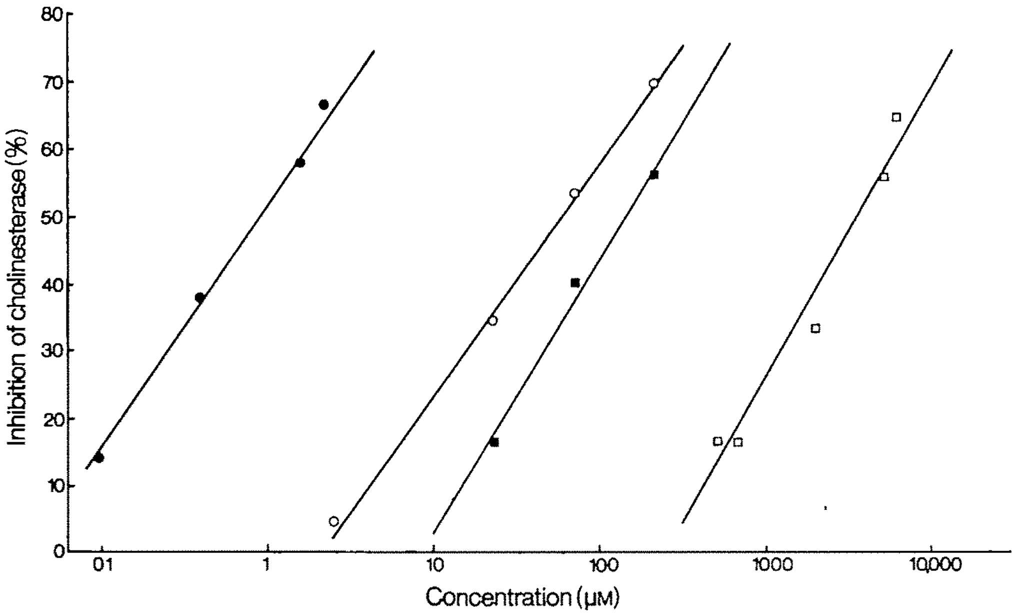

Figure 6 Inhibition of cholinesterase of homogenates of the myenteric plexus-longitudinal muscle preparation. Abscissae, concentration of drug (μM); ordinates, inhibition of cholinesterase (%) The lines have been drawn from regression equations; the number of observations were for physostigmine 41 (●), levorphanol 21 (○), dextrorphan 14 (■), morphine 11 (□).

effects but, in fact, they both improve the contraction, the former without and the latter with a concomitant increase in acetylcholine output.

Since high concentrations of morphine, levorphanol or dextrorphan increase the responses to acetylcholine but not to histamine or bradykinin the phenomena described by Paton are best explained by the anticholinesterase action of high concentrations of morphine.

These observations are probably of little importance for the explanation of tolerance and dependence *in vivo*. The concentrations of morphine required are two to three orders of magnitude higher than the plasma concentrations (1-2 μM) of morphine-dependent guinea-pigs (Goldstein & Schulz, 1973) or the brain concentrations (<1 μM) of morphine-dependent dogs (Mulé & Woods, 1962).

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USE OF THE LOGISTIC FUNCTION FOR THE CALCULATION OF DOSE-RATIOS AND POTENCY RATIOS

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1 Dose-ratios and potency ratios, obtained with the guinea-pig isolated ileum by calculating matching concentrations from a least-squares fit using a logistic relationship between response and dose, differ from those obtained with the more usual assumption that response is a linear function of log dose over a limited range.

2 The differences are not due to the mathematical treatment of the results but arise from changes in sensitivity associated with the production of very high or very low responses.

3 With this preparation there is no advantage in avoiding the linear transformation and fitting the results to the complete dose-response curve though this might not apply to results obtained from other tissues with less variable sensitivity

Introduction

Modern computers make it possible to fit results to many complex expressions directly and thus avoid transforming the results into functions which are linearly related. In the calculation of Michaelis constants from values of substrate concentration and reaction rate, Colquhoun (1971) has shown that the direct fit to the hyperbolic relationship is more accurate as it avoids the bias associated with linear transformations, particularly with the reciprocal-plot method of Lineweaver & Burk (1934).

In most pharmacological tests giving graded responses, such as a contraction of a piece of smooth muscle, it is usual to assume that the response is linearly related to log dose over the middle range of contractions. This linear transformation therefore makes it possible to use only simple algebra for calculating concentrations producing matching responses, such as in bioassay or in the calculation of dose-ratios produced by antagonists or the potency ratios of different agonists. In fact the relationship between log dose and response is usually sigmoid and results can satisfactorily be fitted to the logistic function:

$$\text{response} = M\,\frac{A^p}{A^p + K^p}$$

where A is the concentration of drug, M is the maximal response, and K and p are empirical constants (Parker & Waud, 1971). These authors have described how results may be fitted to this expression for calculating matching responses produced by agonists and partial agonists (or irreversibly blocked agonists) and how these may be fitted to the adsorption isotherm for the calculation of partial agonist (or agonist) affinity constants; it is clear that this procedure should be more accurate than a linear transformation with reciprocals (Barlow, Scott & Stephenson, 1967).

Waud & Parker (1971) have described procedures for fitting sets of results to curves having common values of M and p (i.e. the same maximum response and 'slope') which therefore make it possible to calculate dose-ratios or potency ratios (from the ratios of the values of K). It seemed, therefore, worthwhile investigating whether these were different from values obtained in the usual way, assuming a linear relationship between response and log dose. Ideally the estimations of dose-ratios or potency ratios are null methods, the concentrations being adjusted so that the responses are identical, but in practice the control and test responses invariably differ and it is necessary to make some assumption about the relationship between dose and response in order to calculate matching concentrations. If the difference is of any size, it might be possible that more accurate results would be obtained by fitting the results direct to the logistic function.

Methods

Experiments were made at 37°C with the guinea-pig isolated ileum suspended in aerated

Tyrode solution, containing hexamethonium (2.67×10^{-4} M). Responses were recorded isotonically and the agonist, carbachol, was allowed to act for 30 s and applied once every 90 seconds.

In the first group of experiments dose-ratios were measured for 10^{-6} M phenylacetyltropine methiodide (Abramson, Barlow, Franks & Pearson, 1974). In one set the agonist was tested at two concentrations which produced contractions roughly between 25 and 75% of the maximum, exactly as described previously (Abramson, Barlow, Mustafa & Stephenson, 1969; Edinburgh Staff, 1970). In the second set the agonist was tested in additional concentrations above and below those used in the first set, making four concentrations in all. The order in which they were tested was based on a latin square but because of the way in which the programmer operated the latin square applied to four pairs of concentrations (of the six combinations possible), rather than to the four concentrations themselves.

The mean responses (of five or more observations) to the four concentrations before and after the antagonist were fitted to a logistic expression with common values of M and p and the ratio of the values of K gave the dose-ratio. The mathematical basis for the computation is given by Waud & Parker (1971) and a series of programmes, written in Fortran II, was used to implement the calculations with a PDP 8I digital computer. These required starting estimates of K and p which were then altered until the changes in K were less than 1 in 200.

In the second group of experiments responses were obtained with carbachol, as in the first group, and then a second solution of carbachol was

Table 1

A	*Mean dose-ratio*	*Range*	*s.e.*	n
1	11.6	8.8-15.8	1.1	7
2	9.59	6.5-12.7	0.8	7
3	9.58	6.3-11.9	0.8	7
B	*Mean potency*	*Range*	*s.e.*	n
Standard solution tested first				
1	1.16	1.01-1.29	0.04	6
2	1.39	1.20-1.60	0.06	6
3	1.44	1.31-1.61	0.05	6
Standard solution tested second				
1	1.17	1.02-1.36	0.06	5
2	1.06	0.92-1.14	0.04	5
3	1.00	0.93-1.14	0.04	5
Test conc increased relative to standard; standard solution tested first				
1	1.05	0.90-1.24	0.05	5
2	1.25	1.08-1.46	0.05	5
3	1.29	0.99-1.61	0.13	5
Pooled results: 1	1.13	0.90-1.36	0.03	16
2	1.23	0.92-1.60	0.05	16
3	1.21	0.93-1.61	0.06	16

(A) Dose-ratios produced by phenylacetyltropine methiodide, 10^{-6} M, and (B) potency ratios of carbachol solutions.

1. Experiments with two concentrations of agonist in each set and the results calculated assuming that response is linearly related to log dose. 2. Experiments with four concentrations of agonist in each set and the results calculated from fitting responses to a logistic expression by the method of least squares. 3. Results calculated as in 1 from the responses obtained with the two middle concentrations of agonist used in the experiments in 2.

The concentrations of the carbachol solutions were 1.00×10^{-1} M (standard stock solution) and 1.12×10^{-1} M ('test' stock solution): these were diluted as necessary.

tested. This solution was of known concentration, stored in the same conditions as the stock standard solution of carbachol (4° C), so estimates of its potency by the two methods could be compared and checked with the actual value.

Results

Different dose-ratios were obtained in the two sets (Table 1A). There was no means of knowing the correct value but the results in the second group of experiments (Table 1B) showed that the comparison using four concentrations was an overestimate of potency and in such a situation this would lead to an underestimate of the dose-ratio. When the order of testing the carbachol solutions was reversed, the comparison using four concentrations led to an underestimate of potency, suggesting that the sensitivity of the preparation increased during the experiment so that the solution tested second appeared relatively more active. When the concentrations of the test solution were slightly increased, so that their effects were consistently stronger than those of the standard, the potency ratio was again affected, with the comparison based on two concentrations now apparently an underestimate. These results show that the logistic fit using four concentrations of agonist gives less accurate estimates of potency than does the use of two concentrations and the assumption that response is linearly related to log dose.

Discussion

If the responses with the highest and lowest concentrations of carbachol in the experiments with four concentrations are ignored, an estimate of dose-ratio or potency ratio can be made in exactly the same way as in the experiments with two concentrations of agonist, assuming a linear relationship between response and log dose. These (marked 3 in Table 1) are very similar to the values obtained from the logistic fit of the results with all four concentrations (marked 2 in Table 1). It appears then that, in these experiments, the error arises from the inclusion of the high and low doses in the experimental design rather than from the way in which the results are treated mathematically. The use of concentrations of agonist producing a wide range of responses produces greater changes in sensitivity than when they are confined to the middle range.

In tests with an antagonist the difficulty of ensuring that recovery from its effects is complete usually makes it necessary to obtain separate sets of responses, as in this work, rather than to obtain interspersed responses as is usual in bioassay. In the latter situation the effects of changes in sensitivity should balance out, so the potency ratios obtained with a logistic fit of a wide range of responses should be less inaccurate than found here. With a tissue like the guinea-pig ileum, however, whose sensitivity is variable there would not appear to be any advantage in using a line-fitting procedure with a wide range of responses, even though such a procedure has been shown to be more accurate in other situations.

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THE ESTIMATION OF 3,4-DIHYDROXYPHENYLACETIC ACID, HOMOVANILLIC ACID AND HOMO-ISOVANILLIC ACID IN NERVOUS TISSUE BY GAS–LIQUID CHROMATOGRAPHY AND ELECTRON CAPTURE DETECTION

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1 A gas chromatographic method using electron capture detection is described for the estimation of three acidic metabolites of dopamine, 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid, HVA), 3,4-dihydroxyphenylacetic acid (DOPAC) and 3-hydroxy-4-methoxyphenylacetic acid (homo-isovanillic acid, iso-HVA). The method is based on the formation of the trifluoroacetyl-hexafluoroisopropyl derivatives of the three acids.

2 The method has been applied to the estimation of DOPAC, HVA and iso-HVA in tissues from the central and peripheral nervous systems

Introduction

Methods for the estimation of 3,4-dihydroxyphenylacetic acid (DOPAC) and 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid; HVA) based on the formation of fluorescent derivatives have been used to study the effects of drugs on the metabolism of 3,4-dihydroxyphenylethylamine (dopamine) in the brain. However, these fluorimetric methods are not sensitive enough to enable changes in the concentrations of these two acidic metabolites of dopamine to be measured in small, discrete regions of the central nervous system of laboratory rodents without pooling tissues from several animals. This paper describes a more sensitive method for the estimation of the acid metabolites of dopamine based on the formation of the hexafluoro-isopropyl esters of their trifluoroacetyl derivatives (a procedure first described by Dziedzic, Bertani, Clarke & Gitlow in 1972) and their estimation by gas-liquid chromatography (GLC) combined with electron capture detection.

Methods

Materials

Ethyl acetate: Reeve Angel Scientific Ltd, CT grade. If required dry, this was dried over calcium hydride and distilled twice.

Trifluoroacetic anhydride (TFA): BDH Chemicals Ltd. This reagent was distilled twice using a 10 cm fractionating column filled with Fenske helices and was then stored at −17° C in sealed glass ampoules each containing sufficient trifluoroacetic anhydride for one set of samples (usually 1.5 ml). Any reagent remaining in an ampoule was discarded.

1,1,1,3,3,3-Hexafluoroisopropanol (HFIP): Pierce Chemical Co. This reagent was distilled once using a 20 cm long Fenske helix fractionating column and stored under dry N_2 at room temperature.

Diethyl ether: Reeve Angel Scientific Ltd, CT grade. Boron trifluoride 99.5%: BDH Chemicals Ltd.

Boron trifluoride etherate. Approximately 1 ml diethyl ether was placed in a test tube and boron trifluoride was passed into it through a polytetrafluoroethylene tube until the diethyl ether was saturated.

4-Hydroxy-3-methoxyphenylacetic acid (homovanillic acid; HVA): Calbiochem and Sigma. 3-Hydroxy-4-methoxyphenylacetic acid (homo-isovanillic acid, iso-HVA), prepared by the method of Grundon & Perry (1954), m.p. 129.5-130° C uncorrected. 3,4-Dihydroxyphenylacetic acid (DOPAC): Sigma, recrystallized from benzene.

Pentafluorophenol: Koch-Light Ltd. Distilled once under vacuum.

Benzoyl chloride: May & Baker Ltd.

Other chemicals were of analytical reagent quality.

Gas-liquid chromatography

This was carried out with Pye model 104 gas-liquid chromatographs fitted with [^{63}Ni] electron capture detectors. The carrier gas was argon containing 5% or 10% methane. Several liquid phases coated on Diatomite CQ were tested and these together with column and detector oven temperatures and gas flow rates are given with the results. These columns were cured at 250° C with the carrier gas flowing through them for at least 16 h before use.

Reaction vials

The bases of small screw capped glass vials (0.5 dram series SNB, Camlab Ltd) were blown to form round bottomed reaction vials. The plastic caps were fitted with a disk of clear silicone rubber (Esco Rubber) 1.5 mm thick and a disk of polytetrafluoroethylene 0.25 mm thick which ensured an inert gas-tight seal.

Evaporations

These were always carried out in a thermostatically controlled metal heating block.

Preparation of crystalline derivatives of HVA, DOPAC and iso-HVA

Approximately 0.5 g of the parent acid was added to a mixture of 5 ml trifluoroacetic anhydride and 2.5 ml hexafluoroisopropanol. This was usually refluxed overnight but a reasonable yield of the HVA derivative can be obtained after 5 hours. The trifluoroacetic anhydride and hexafluoroisopropanol were removed at room temperature under vacuum and the remaining liquid was distilled under vacuum. The HVA derivative distilled at 120-130° C at approximately 8 mmHg pressure (water pump). The product was a clear, colourless, viscous liquid which crystallized when stored at −17° C but was liquid near room temperature (m.p. 27-30° C uncorrected). The DOPAC derivative distilled at 84-88° C at 0.1 mmHg pressure yielding a clear colourless viscous liquid which crystallized on cooling (m.p. 44-45° C uncorrected). The iso-HVA derivative distilled at 95-97° C at 0.3 mmHg pressure and also gave a clear, colourless, viscous liquid which crystallized when stored at −17° C and melted at 31-33° C (uncorrected).

Preparation of pentafluorophenyl benzoate

Pentafluorophenyl benzoate (PFPB) was found to be a suitable substance to serve as a standard in determining the concentrations of DOPAC, HVA and iso-HVA in tissue extracts by the peak height ratio method (Vandenheuvel, Hinderks, Nixon & Layng, 1965; Wisniewski & Umbreit, 1965).

Pentafluorophenol 1.0 g was dissolved in 5 ml 10% w/v NaOH solution cooled in an ice bath; 1.0 ml benzoyl chloride was added slowly and the mixture then shaken vigorously for 5-10 min with intermittent cooling. A white semi-crystalline solid formed and was extracted into dichloromethane. The extract was dried over anhydrous sodium sulphate and evaporated to dryness under vacuum. The white crystalline solid which remained was dissolved in warm hexane, the solution was filtered and then cooled to 0° C. The crystals (m.p. 73-75° C uncorrected) which formed were filtered off in a cold filter funnel and then dried on filter paper until no smell of benzoyl chloride could be detected.

Extraction of tissues

Two methods of precipitating tissue proteins have been used. The first, using zinc sulphate and barium hydroxide, was found to be suitable for the estimation of HVA alone and the second, using perchloric acid followed by potassium chloride to remove the perchlorate ion from solution, enabled the estimation of iso-HVA and DOPAC in addition to HVA.

Method 1. The estimation of HVA in nervous tissues

Up to 250 mg tissue was frozen in liquid nitrogen, crushed in a stainless steel die if necessary and then homogenized in 1.0 ml 0.2 M zinc sulphate solution. The homogenate was transferred to a centrifuge tube together with 1 ml water used to wash the homogenizer. To this, 1.0 ml of 0.2 M barium hydroxide solution was added and mixed thoroughly. (The zinc sulphate and barium hydroxide solution must be titrated against each other before use with phenol phthalein as indicator, to ensure equal normalities.) The mixture was centrifuged and the clear supernatant was transferred to a glass test tube containing 0.5 g potassium chloride and 0.1 ml 1 N HCl. Ethyl acetate (4.0 ml) was added and the HVA extracted by mixing for 1 min on a vortex mixer. The phases were separated by centrifugation and 3.5 ml of the ethyl acetate was taken and evaporated under dry N_2 at 50° C to a volume of approximately 0.2 ml. This was transferred to a reaction vial and the tube washed with approximately 0.1 ml ethyl acetate which was also added to the vial. The ethyl acetate extract was then evaporated to dryness under dry N_2 at 50° C. The vial was removed from the heating block as soon as all of the ethyl acetate had disappeared; 0.2 ml of trifluoroacetic anhydride was added to the reaction vial followed by 0.1 ml hexafluoroisopropanol containing 10% v/v freshly prepared boron trifluoride etherate. The reaction vial was tightly closed, the contents mixed and it was then heated at 100° C in a metal heating block for 15 minutes. The estimation was unsatisfactory if the reaction vial leaked during this heating. The reaction vial was then allowed to cool to room temperature before opening and the contents were evaporated under a stream of dry N_2 at room temperature until a small amount of an oily brown residue remained. This was dissolved in 0.5-1.0 ml of dry ethyl acetate containing a known concentration of pentafluorophenyl benzoate (20-50 ng/ml).

In the early experiments to determine the optimal conditions for the reaction, it was observed that a low recovery of the derivative of HVA was obtained if the boron trifluoride etherate was omitted from the reaction. However, it was later found with tissue extracts that a satisfactory recovery could be obtained when the boron trifluoride was omitted if the heating was continued for 60 min and the final evaporation was stopped as soon as all of the liquid had disappeared. Most of the results have been obtained without using boron trifluoride etherate because fewer peaks are seen on the chromatograph tracings when this reagent is omitted. It seems that the presence of the boron trifluoride etherate resulted in a material with a high boiling point which retained the volatile HVA derivative in the reaction vial thus preventing the loss seen with pure solutions which was presumably due to evaporation of the HVA derivative.

Method 2. The simultaneous estimation of HVA and DOPAC

The procedure now used routinely is as follows·

1. Dissect tissue (up to 250 mg) and freeze at once in liquid N_2. Crush in stainless steel die if necessary.
2. Homogenize in 1.0 ml 0.1 N HCl, add 1.0 ml H_2O and mix.
3. Add 25 μl 72% w/v perchloric acid; mix.
4. Add 0.5 g KCl, mix and then centrifuge in homogenizer tube at 5,000 x *g* for 3 min at room temperature.
5. Carefully transfer (avoid transfer of fatty material) the supernatant to a cellulose nitrate centrifuge tube containing 0.5 g KCl; mix.
6. Freeze in liquid N_2 until solid. Allow to thaw until liquid is just visible around a frozen pellet. Centrifuge at 15,000 x *g* for 15 min at 10° C. This step was found to be necessary for consistent recoveries.
7. Transfer supernatant to a glass test tube containing a few crystals of KCl. Add 4.0 ml ethyl acetate and mix on a vortex mixer for two periods of 30 s each. The tubes are placed in ice between the two mixes while the contents of the other tubes in the series are mixed. Centrifuge at 4,000 x *g* for 1 min to separate the phases.
8. Transfer 3.5 ml of the organic upper layer to a glass test tube and evaporate at 50° C under a stream of dry N_2 until approximately 0.2 ml remains. This is transferred to a reaction vial and the tube washed with 0 1 ml ethyl acetate which is also transferred to the reaction vial. Evaporate the ethyl acetate to dryness at 50° C under a stream of dry N_2.
9. Add 0.2 ml trifluoroacetic anhydride and 0.1 ml hexafluoroisopropanol to the reaction vial and close tightly. Heat at 100° C for 1 hour.
10. Allow reaction vial to cool to room temperature before opening and then evaporate contents *just* to dryness at room temperature under a stream of dry N_2. Dissolve the residue in a measured volume of dry ethyl acetate containing a known amount of pentafluorophenyl benzoate for injection into the gas chromatograph. Routinely the residue is dissolved in 1.0 ml of dry ethyl acetate containing 50 ng/ml pentafluorophenylbenzoate; 0.2-2 μl of this solution is injected into the gas chromatograph.

Extraction of cerebrospinal fluid and vitreous humour

Pig cisternal cerebrospinal fluid (0.5 ml) was diluted to 2 ml with 0.1 N HCl in a cellulose nitrate centrifuge tube; 25 μl perchloric acid was added and mixed, followed by 1.0 g potassium chloride. After thorough mixing to ensure saturation with the potassium chloride the solution was frozen solid in liquid N_2, partially thawed, centrifuged at 15,000 x *g* for 15 min and the supernatant treated as above. Aqueous and vitreous humour from goat and pig eyes was extracted in a similar manner, the amounts of reagents used in the extraction of the phenolic acids being increased approximately in proportion to the volume of body fluid used.

Results

The retention times of the TFA-HFIP derivatives of DOPAC, HVA and iso-HVA relative to that of PFPB are shown in Table 1 and show that clear separations of the derivatives can be obtained. The ratios of the peak heights of the derivatives of DOPAC and HVA to that of PFPB showed a linear relation with the amounts of the derivatives of DOPAC and HVA in a sample (prepared from crystalline derivatives) relative to the amount of PFPB present in the same sample. Thus the peak height ratio method could be used to quantify the amounts of HVA and DOPAC present in extracts of tissues.

The recovery of HVA (50 ng) added to homogenates of mouse brain and estimated after protein precipitation with zinc sulphate and barium hydroxide gave a mean recovery (±s.e. mean) of 79 ± 6% ($n = 28$) when estimated with a 3.8% S.E.30 column and 65 ± 5% ($n = 28$) when the same samples were estimated with a 2% QF1 column It was found that of the three types of column tested, that with a stationary phase of 2% S.E.52 was the most suitable for the simultaneous estimation of HVA and DOPAC. The recovery of DOPAC and HVA (25-50 ng) added to homogenates of mouse brain tissue and estimated with a 2% S.E.52 column was 62 ± 3% (mean ± s.e. mean; $n = 51$) and 65 ± 4% ($n = 59$) respectively.

The reproducibility of the method was tested by estimating the concentration of HVA and DOPAC in replicate portions of homogenates of mouse striatal tissue. Most duplicate estimates have shown reasonable reproducibility and in 11 experiments in which the estimation of HVA was made in triplicate or quadruplicate the mean coefficient of variation was 8.9% (range 0.9-14.8%) and in two experiments in which DOPAC was estimated in triplicate the coefficients of variation were 11.8% and 12.9%.

The specificity of the method has been tested by estimating the concentration of HVA in mouse brain tissue with two different chromatography columns and by comparing the values obtained by the GLC method for the concentrations of HVA and DOPAC in different tissues with the values which have been obtained by fluorimetry.

Table 2 gives estimates of the concentrations of DOPAC and HVA obtained with the present GLC method in different nervous tissues and body fluids.

When the HVA in the striatal tissue of mice was

Table 1 The retention times of the trifluoroacetyl-hexafluoroisopropyl (TFA-HFIP) derivatives of acid metabolites of dopamine relative to that of pentafluorophenyl benzoate

Column length and liquid phase	*Column temperature (°C)*	*Relative retention time*		
		TFA-HFIP DOPAC	*TFA-HFIP HVA*	*TFA-HFIP iso-HVA*
2.75 m, 2% S.E. 52	115	0.35	0.52	0.58
	95	0.37	0.55	0.63
1.5 m, 2% QF 1	105	1.52	1.36	
1.5 m; 3.8% S.E. 30	105	0.36	0.52	

The carrier gas was argon containing 5% or 10% methane with a flow rate of 40 ml/min at pressures of 15-20 kg/cm². The detector temperature was 220°C

Table 2 The concentration of 3,4-dihydroxyphenylacetic acid (DOPAC) and 4-hydroxy-3-methoxyphenylacetic acid (HVA) in some tissues and body fluids

Species	*Tissue or body fluid*	*Concentration (μg/g or *μg/ml)*	
		DOPAC	*HVA*
Rat	Caudate nucleus	2.26 ± 0.37 (9)	0.58 ± 0.06 (9)
	Olfactory tubercle	1.14 ± 0.15 (5)	0.52 ± 0.04 (5)
Mouse	Striatum	0.17 ± 0.01 (13)	0.26 ± 0.02 (13)
Pig	Cisternal cerebrospinal fluid	0.014 ± 0.002 (4)*	0.060 ± 0.001 (4)*
Goat	Vitreous humour	0.038; 0.085*	0.021, 0.012*
	Retina	0.008; 0.005†	0.004; 0.004†
Rabbit	Superior cervical ganglion	not estimated	0.42 ± 0.04 (4)
Sheep*	Caudate nucleus	0.94 ± 0.18 (3)	6.7 ± 1.8 (3)

Values are given as mean with s.e. mean and are uncorrected for recovery. The number of observations is in parentheses. † Content in μg/retina, 2-4 retinae were pooled for each estimation.

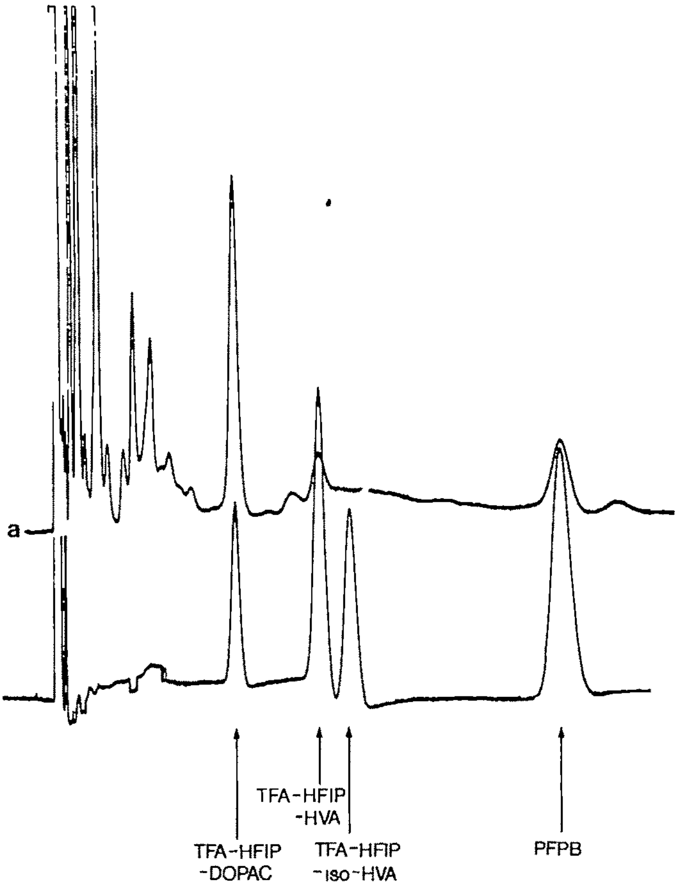

Figure 1 Gas chromatographic record obtained with (a) an extract of rat striatum compared with (b) standards. The arrows indicate the retention times of the trifluoroacetyl-hexafluoroisopropyl derivatives of 3,4-dihydroxyphenylacetic acid (TFA-HFIP-DOPAC), 4-hydroxy-3-methoxyphenylacetic acid (TFA-HFIP-HVA), 3-hydroxy-4-methoxyphenylacetic acid (TFA-HFIP-iso-HVA) and pentafluorophenyl benzoate (PFPB). The column was 2.75 m long packed with 2% S.E.52 coated on Diatomite CQ. Oven temperature 115° C. Detector temperature 220° C. Carrier gas argon· 5% methane; flow rate 40 ml/min at 20 kg/cm^2.

estimated on both the 3.8% S.E.30 and 2% QF1 columns the values obtained were not significantly different ($t = 0.25$; $n = 8$; paired t test) although the relative retention times differ greatly on the two columns. The estimates of the concentrations of HVA and DOPAC in the mouse striatum given in Table 2 were obtained with 2% S.E.52 columns. The values obtained compare well with those reported by Sharman (1966, 1967) for HVA and Roffler-Tarlov, Sharman & Tegerdine (1971) for DOPAC, and were similar to the values obtained in this laboratory by fluorimetric methods during the period when the GLC estimates were made. A chromatograph record of an extract of rat striatum is given in Figure 1. The present estimates for the concentrations of the two acids in the rat caudate nucleus confirm the observations of Guldberg & Broch (1971) that the major metabolite of dopamine present in rat striatal tissue is DOPAC.

Although both HVA and DOPAC could be estimated in the vitreous humour of the goat, the application of the GLC method to aqueous humour met with a major difficulty. There was a substance present in extracts of aqueous humour which had a retention time relative to PFPB very close to that of the derivative of DOPAC. However, no DOPAC (<10 ng/ml) could be detected in the same sample of aqueous humour when a fluorimetric method (Murphy, Robinson & Sharman, 1969) was used for its estimation. No HVA could be detected in aqueous humour.

The GLC method will also permit the concurrent estimation of iso-HVA (Dziedzic, Dziedzic & Gitlow, 1973; Dziedzic & Gitlow, 1974). However, in all of the samples which we have examined, except for sheep caudate nucleus, no evidence for the presence of iso-HVA was obtained. With extracts of sheep caudate nucleus small peaks were observed with a relative retention time identical with that of TFA-HFIP-iso-HVA. These gave an estimate of the concentration of iso-HVA in sheep caudate nucleus of 0.8 ± 0.3 μg/g ($n = 3$), equivalent to 12% of the concentration of HVA in this tissue.

Discussion

The present method for the estimation of HVA and DOPAC is applicable to a wide range of tissues. Dziedzic *et al.* (1973) and Dziedzic & Gitlow (1974) have demonstrated that the GLC method is suitable for the estimation of HVA in human urine and cerebrospinal fluid and the present experiments show that it is possible to extend this to the estimation of DOPAC.

The limit of sensitivity is about 1 ng of HVA or DOPAC in a tissue sample which is similar to that reported for HVA in cerebrospinal fluid by Dziedzic & Gitlow (1974). However, like most methods for the estimation of very small amounts of substances its specificity, although good, is not absolute. This is illustrated by the presence of an unknown substance in extracts of aqueous humour which might be mistaken for DOPAC. It is thus advisable always to examine extracts of tissues using a second chromatography column on which the relative retention times of the derivatives are different and preferably show a different sequence of elution or to examine a pooled sample of the tissue under investigation by fluorimetry.

The method allows studies of the formation of the acid metabolites of dopamine in smaller samples of brain tissue than has been possible by fluorimetric techniques and may enable more precise observations to be made on the role of dopamine in the central nervous system.

The only tissue in which iso-HVA was detected was the sheep caudate nucleus. This confirms the observation of Mathieu, Charvet, Chazot & Trouillas (1972) that iso-HVA is present in the sheep caudate nucleus. The present estimate of the concentration (12% of the HVA present) is much lower than that found by their chemical method of assay (53-58%), however, the three sheep examined were of different breeds and the concentrations varied widely.

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THE EFFECT OF OXYTOCIN ON BLOOD VESSELS OF CREMASTER MUSCLE OF THE RAT

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Oxytocin administered intravenously to the anaesthetized rat produced dilatation of the blood vessels of the cremaster muscle at concentrations ranging from 2.5×10^{-11} to 2.5×10^{-9} M When applied topically to the exposed vessels it produced constriction at concentrations ranging from 2.5×10^{-12} to 5.0×10^{-8} M. Oxytocin was thus similar to adrenaline in eliciting opposite effects when applied to the serosa or to the intima of skeletal muscle blood vessels.

Introduction It is known from clinical observation that administration of exogenous oxytocin to women can cause flushing of the skin and a measurable fall in blood pressure. Pickford and her colleagues (Pickford, 1966) showed that in man this response occurred in skin and muscle vessels of the limbs, and that it could be converted to vasoconstriction by sympathectomy or by the presence of circulating ovarian hormones. Using male rats Honoré & Lloyd (1961) observed dilatation of the vessels of the mesoappendix in response to intravenous injection of 75 mu doses of oxytocin; but Berde (1965) was unable to record any difference from controls in the ^{86}Rb content of diaphragm and gastrocnemius muscles after infusion of oxytocin, i.e., he found no increase in blood flow after oxytocin. It was considered that Grant's (1964) technique of observing the blood vessels of rat cremaster muscle under the dissecting microscope might reveal more directly how the muscle blood vessels respond to oxytocin. Grant was satisfied that, in respect of all the substances which he used, the vessels in the cremaster muscle responded in the same way as blood vessels of the musculature of the thigh, and he considered that cremaster vessel responses might be taken to be representative of responses of 'limb skeletal muscles in general'.

Methods Young male rats (170-200 g) were anaesthetized with urethane, the jugular vein was cannulated, and a slow infusion of gelatin-Ringer was initiated at a rate of about 0.5 ml/h; the cremaster muscle was exposed and irrigated with gelatin-Ringer at 30-31° C for examination of the blood vessels under the dissecting microscope as described by Grant (1964). When the effect on the blood vessels of intravenous administration of hormones or drugs was to be examined, the material was injected into the venous cannula. When the effect of topical application was to be examined, the material was added to the pool of gelatin-Ringer irrigating and bathing the cremaster muscle, the volume of this pool being estimated to be 0.5 ml. The effect of each dose treatment was examined in at least five animals. The validity of the method was confirmed by repeating Grant's observations on the effects of histamine, adrenaline, noradrenaline and acetylcholine. In all experiments the sensitivity of the preparation was confirmed by observing the dilatation of the blood vessels produced by a small intravenous dose of adrenaline (usually 5 µg/rat). Solutions for injection or irrigation were prepared in gelatin-Ringer. Oxytocin was Pitocin (Parke Davis) having oxytocic activity 10 u/ml and containing chlorbutol 0.5%.

Results Intravenous administration of oxytocin in doses ranging from 0.1 mu to 10 mu always produced dilatation in the cremaster muscle blood vessels, with onset about 1 min (range 0.5 to 3 min) after injection. This dilatation was recorded as vessels becoming 'clearer', 'more distinct', 'redder' or 'wavy'. Branches appeared on the large vessels, and small vessels opened up and became visible where they had been previously constricted and invisible. Both arterial and venous vessels became dilated. Usually a clear dilatation was still visible at 5 to 10 min and thereafter a slow recovery took place, and the normal resting appearance was regained at about 15 minutes. Occasionally, along with the general dilatation, or following it, constriction was observed in single arterial branches. When injections were repeated the recovery from dilatation took place more quickly, and in some animals after repeated injections the response to the smallest dose could no longer be detected. Table 1a shows the doses used. Assuming that injected material would be dispersed in a plasma volume of about 10 ml, the concentration of oxytocin reaching the intima of the blood vessels was calculated and is shown in Table 1a. Dilatation of the vessels was produced by concentrations of oxytocin down to 2.5×10^{-11} M. Since rat pituitaries were found on

Table 1 Effect of drugs on small blood vessels of rat

Vascular bed	*Surface exposed to drug*	*Treatment*	*Estimated concentration of drug (M) applied to blood vessels*	*Observed effect*
(a) Cremaster muscle	Intima	*Oxytocin*		
		Intravenous 0.1 mu	2.5×10^{-11}	D
		Intravenous 1.0 mu	2.5×10^{-10}	D
		Intravenous 4.0 mu	1.0×10^{-9}	D
		Intravenous 10.0 mu	2.5×10^{-9}	D
(b) Cremaster muscle	Serosa	*Oxytocin*		
		Irrigation of tissue 1.0 μu/ml	2.5×10^{-12}	C
		Irrigation of tissue 10.0 μu/ml	2.5×10^{-11}	C
		0.1 mu added to 0.5 ml pool	5.0×10^{-10}	C
		1.0 mu added to 0.5 ml pool	5.0×10^{-9}	C
		10.0 mu added to 0.5 ml pool	5.0×10^{-8}	C
(c) Mesoappendix	Intima	*Oxytocin* (Lloyd, 1959)		
		Intravenous 10-30 mu	10^{-9}-10^{-8}	D
(d) Mesoappendix	Serosa	*Oxytocin* (Lloyd, 1959)		
		Bathed with 50 mu/ml	1.0×10^{-7}	D
(e) Cremaster muscle	Intima	*Adrenaline* (Grant, 1964)		
		Intravenous 0.01 to 0.1 μg	10^{-9}-10^{-8}	D
(f) Cremaster muscle	Serosa	*Adrenaline* (Grant, 1964)		
		Local application 0.01-10.0 ng/ml	10^{-11}-10^{-7}	C

D = dilatation, C = constriction
Sections (a) and (b) are the results of the present investigation; sections (c) and (d) are from Lloyd (1959); sections (e) and (f) are from Grant (1964).

assay to have approximately 500 mu of oxytocic activity, it could be calculated that the smallest effective dose of oxytocin was equivalent in terms of oxytocic activity to the addition to the circulating plasma of about 1/5000 of a rat pituitary. Control injections were made with equivalent volumes of gelatin-Ringer, and gelatin-Ringer with chlorbutol (1.0 μg and 5.0 μg), and none of these injections produced any detectable effect on the blood vessels.

Topical application of oxytocin in doses ranging from 0.5 μu to 10 mu added to the 0.5 ml pool of gelatin-Ringer bathing the cremaster muscle always produced vasoconstriction of the blood vessels. The lower doses of oxytocin were applied by having a double irrigation system, and alternating between irrigation with gelatin-Ringer and irrigation with gelatin-Ringer containing oxytocin. Onset of vasoconstriction was seen only 1 to 1.5 min after the irrigation with oxytocin had been started, and if the irrigation was continued the vasoconstriction passed off after about 5 to 10 minutes. When irrigation with oxytocin had produced an obvious vasoconstriction, then changing to gelatin-Ringer hastened the recovery to normal. The higher doses of oxytocin were applied by gently adding the oxytocin, suitably diluted in gelatin-Ringer, to the pool of gelatin-Ringer surrounding the muscle. Onset of vasoconstriction was usually recorded at about 1 minute. It was frequently noted that constriction would begin at one end of a large vein, and would slowly spread along the visible length of it; irrigation with gelatin-Ringer continuing, the vasoconstriction began to diminish after about 5 min, and the appearance returned to normal at about 10 minutes. Table 1b shows the doses and concentrations of oxytocin which produced vasoconstriction on topical application.

Discussion It is notable that in the present experiments very small intravenous doses of oxytocin, producing plasma concentrations of approximately 2.5×10^{-11} M, have been shown to dilate the blood vessels of the rat cremaster muscle. The possible dilator effect of the chlorbutol, also present in the solutions, must be considered, as has been discussed by Somlyo, Woo & Somlyo (1966). The highest dose of oxytocin used (10.0 mu) would be accompanied by 5.0 μg of chlorbutol. Control injections showed that this amount of chlorbutol never produced vasodilatation; and when diluted in the plasma it would give a concentration of approximately 0 5 μg/ml which is far below the threshold concentration of 0.1 mg/ml which Somlyo *et al.* (1966) recorded for arterial smooth muscle. The possibility of the observed dilatation being due to chlorbutol is thus ruled out. Lloyd (1959) observed that intravenous injection of oxytocin produced dilatation in the vessels of the rat mesoappendix, the threshold dose being 10 mu/rat (Table 1c). This concentration was of a different order from that in the human experiments of Deis, Kitchin & Pickford (1963) where intravenous injection of 500 mu oxytocin was shown to produce increased hand blood flow, and the concentration of oxytocin reaching the microvasculature would have been approximately 2.5×10^{-10} M. If we accept Grant's (1964) demonstration that the response of the cremaster muscle vessels is characteristic of skeletal muscle vessels in general, then the present results might be taken to indicate that in the rat, just as in man, skeletal muscle vessels are dilated by oxytocin in concentrations of the order of 10^{-10} M.

Topical application of oxytocin to the exposed tissue of the cremaster muscle produced results which were different from those seen by Lloyd (1959) in the mesoappendix. Lloyd observed dilatation (Table 1d) whereas in the present experiments (Table 1b) vasoconstriction was always seen. The present findings agree with the observations of Altura (1972); in his experiments oxytocin topically applied produced constriction in the metarterioles of rat mesentery, with a threshold concentration of 10^{-8} M and a dose-dependent increase to a maximum at 5×10^{-5} M.

The observed difference in the response to oxytocin according to whether it is applied to the intima or to the serosa of the microvasculature is reminiscent of Grant's (1964) observations on adrenaline (Table 1e and 1f). The difference might derive, as has been suggested by Altura & Hershey (1967) from the different responses of arterioles, venules and capillaries, or perhaps, as suggested by Grant with reference to adrenaline, from the anatomical situation of the receptors in the smooth muscle of the vessels. The only previous indication of such a difference in respect of oxytocin seems to be in the work of Altura & Hershey (1967) who noted that topically applied oxytocin constricted the metarterioles of rat mesentery, while if intravenously administered it depressed the contractile response to catecholamines.

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KINETICS AND SOME CHARACTERISTICS OF UPTAKE OF NORADRENALINE BY THE HUMAN UMBILICAL ARTERY

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The uptake of exogenously added noradrenaline (NA) (0.5-2.5 μg/ml) by the human umbilical artery was linear with time up to 10 minutes. The uptake was saturable and could be described by the Michaelis-Menten equation The uptake was cocaine-resistant, normetanephrine-sensitive, was considerably inhibited in the cold and was partially inhibited by Na^+-deficiency. Of NA accumulated in the artery 31% could be washed out by NA-free medium It is concluded that the mechanism of uptake of NA by the human umbilical artery is similar to the $uptake_2$ mechanism.

Introduction In innervated tissues, $uptake_1$ and the presence of metabolizing enzymes, catechol-*O*-methyltransferase (COMT) and monoamine oxidase (MAO) can interfere with the kinetic analysis of extraneouronal uptake of noradrenaline (NA) The human umbilical artery lacks adrenergic innervation and the MAO and COMT activity of this tissue is very low (Burnstock, McCulloch, Story & Wright, 1972). In the present study the kinetics and some characteristics of uptake of NA were determined using the human umbilical artery.

Methods Umbilical cords were obtained immediately after delivery from the Maternity Ward of Shree Sayaji General Hospital, Baroda and kept in the medium described below. After dissecting out both the arteries the lumen of each artery was cut open and 6 pieces each weighing about 100-200 mg were prepared from both arteries. Unless otherwise specified, all preincubations or incubations were carried out at 37°C in 30 ml of the medium (g/l NaCl 9.0, KCl 0.42, $CaCl_2$ 0.24; $NaHCO_3$ 0.2; disodium edetate 0.01, and glucose 1.0) bubbled with 5% CO_2 in O_2 (pH 7.4). Preincubations and incubations were carried out in separate flasks. The Na^+-deficient medium contained sucrose (105.3 g/l) and $KHCO_3$ (0.238 g/l) instead of NaCl and $NaHCO_3$ respectively. NA was measured fluorometrically (Anton & Sayre, 1962). Fluorescence was read on a Turner fluorometer (Model 110). The mean recovery of NA added to alumina was 70 ± 2.5% (n = 10) and all values were corrected for 70% recovery.

For studying the kinetics of uptake four pieces of artery were preincubated for 10 min in individual flasks and then incubated with specified concentrations of NA for different times. The effects on uptake of the following drugs or procedures were studied· (i) preincubation of the artery for 10 min with normetanephrine (10 μg/ml) or cocaine (5 μg/ml) followed by incubation for 5 min with a specified concentration of NA and either normetanephrine (10 μg/ml) or cocaine (5 μg/ml), (ii) preincubation with Na^+-deficient medium with change of the medium every 10 min three times followed by incubation for 5 min in Na^+-deficient medium containing NA (1 μg/ml); (iii) preincubation with NA-free medium for 10 min, incubation for 5 min with medium containing NA (1 μg/ml) followed by incubation with NA-free medium with change of medium every 3 min, (iv) preincubation for 15 min at 4°C followed by incubation for 5 min at 4°C with medium containing NA (1 μg/ml).

For determination of the extracellular space, a piece of the artery was preincubated in the medium for 10 min and then incubated with alkali stable inulin (100 μg/ml) for specified times. The inulin content of the artery was estimated by the method of Schneyer & Schneyer (1960). The extracellular space (ml/g) of the artery was derived by the formula inulin content per g of artery/inulin concentration per ml of incubation medium. The mean extracellular space values after incubation with inulin for 10, 20, 30 and 60 min were 0.23 ± 0.03, 0.3 ± 0.02, 0.37 ± 0.09 and 0.38 ± 0.02 ml/g respectively (n = 3 each). The values for 30 and 60 min are closely similar and, therefore, the 30 min value was used for correcting for NA present in the extracellular space. In washout experiments no correction was made for extracellular space.

The drugs used were: cocaine hydrochloride (May & Baker), (−)-noradrenaline (NA) (Rhone Poulenc); (±)-normetanephrine hydrochloride (Sterling-Winthrop). The concentrations of NA are in terms of base and those of other drugs are in terms of salt.

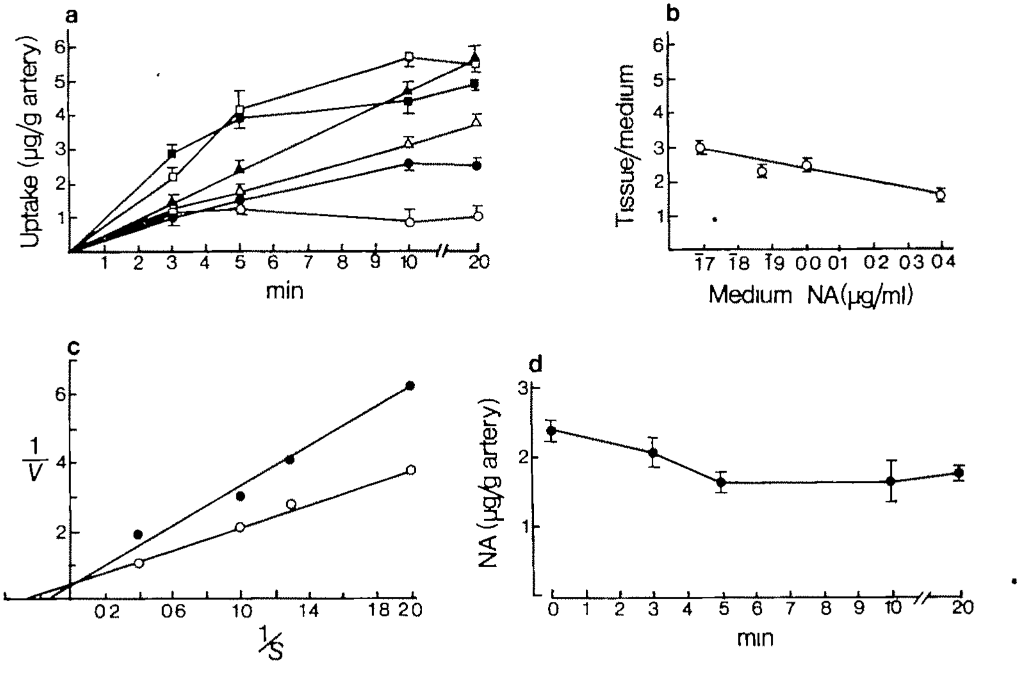

Figure 1 (a) The uptake of noradrenaline (NA) by the human umbilical artery incubated with 0.25 µg/ml (○), 0.5 µg/ml (●), 0.75 µg/ml (△), 1 µg/ml (▲), 2.5 µg/ml (□) and 5 µg/ml (■) of NA for different times. Each point is the mean of 3 values. (b) Five min data of (a) except for the lowest and the highest concentrations. The final medium concentration of NA is plotted against the ratio of tissue concentration vs. the final medium concentration of NA. (c) Kinetic analysis of NA uptake (data plotted according to regression analysis). S = concentration of NA in incubation medium (µg/ml). V = rate of uptake (µg g^{-1} min^{-1}) For control (○), the 5 min data as in (b) were used. (●) Represents the effect of normetanephrine (10 µg/ml, $n = 3$) (d) Effect of wash out with NA-free medium on the NA accumulated in the artery following incubation with NA (1 µg/ml) for 5 minutes The time of incubation with NA-free medium is on the abscissa scale and the NA content of the artery is on the ordinate scale. Each point is the mean of three values. Vertical lines in (a), (b) and (d) indicate s.e. mean.

Results No endogenous NA could be detected in the human umbilical artery ($n = 10$) confirming the observation of Burnstock *et al.* (1972) that this tissue lacks adrenergic innervation.

The uptake of NA within the concentration range of 0.5-2.5 µg/ml of NA in the medium was linear with time up to 10 minutes. With lower and higher concentrations and beyond 10 min, the relationship was not linear (Figure 1a). The plot of tissue/medium concentration ratio vs. medium concentration indicates that the uptake was saturable (Figure 1b). The apparent K_m and V_{max} values obtained from a plot of $1/V$ against $1/S$ were 3.3 µg/ml (1.95 x 10^{-6} M) and 2.0 µg min^{-1} g^{-1} artery (0.0121 µmol min^{-1} g^{-1}) respectively (Figure 1c). Normetanephrine inhibited the uptake of NA and in its presence a linear relationship was observed between the plot of $1/V$ against $1/S$ (Figure 1c). The ordinate intercept for this line and the control line was common. The K_p value (i.e. the K_m of the substrate in the presence of the inhibitor) was 3.7 x 10^{-6} M. The K_i value of normetanephrine was 5.7 x 10^{-5} M.

The mean control uptake at the end of 5 min incubation with NA 1 µg/ml was 2.43 ± 0.15 µg/g ($n = 3$). The mean uptake in the presence of cocaine was 2.8 ± 0.16 µg/g ($n = 3$, $P > 0.5$). At 4°C, the mean uptake was 0.82 ± 0.02 µg/g ($n = 3$; $P < 0.01$). In Na^+-deficient medium, the mean uptake was 1.09 ± 0.33 µg/g ($n = 6$; $P < 0.01$).

Washing with NA-free solution was associated with reduction in the amount of NA accumulated, the maximum effect (31%; $P < 0.05$) being observed at 5 min (Figure 1d).

Discussion The uptake of exogenously added NA by the human umbilical artery was linear up to 10 min within the concentration range of

0.5-2.5 μg/ml, was saturable and was reduced by 66.3% of control in the cold, indicating that it was largely an active process. The uptake could be described by the Michaelis-Menten equation. The apparent K_m and the V_{max} values obtained by us are lower than the respective values (2.52×10^{-4} M and 0.102 μmol min^{-1} g^{-1}) obtained by Iversen (1965) for the isolated innervated rat heart; the lower values could be ascribed to the lack of adrenergic innervation and the very low activity of the enzymes MAO and COMT (Burnstock *et al.*, 1972) and are in accord with the observation that the uptake_2 of NA in innervated tissues occurs at low concentrations when the enzymes MAO and COMT are inhibited (Lightman & Iversen, 1969).

Cocaine, a potent inhibitor of uptake_1, was inactive while normetanephrine a potent inhibitor of uptake_2 (Iversen, 1967) inhibited the uptake competitively. The uptake was partially Na^+-dependent. Gillespie & Towart (1973) similarly demonstrated that the extraneuronal uptake of NA in the smooth muscle of the rabbit ear artery is partially Na^+-dependent. Finally 31% of NA accumulated in the umbilical artery could be washed out. The partial effect of washing out may be due to intracellular binding of NA (Burnstock, McLean & Wright, 1971).

In certain respects our results are at variance with those of Burnstock *et al.* (1971) e g. they did not observe any inhibition of uptake of NA by the human umbilical artery with normetanephrine and in the cold. Gillespie, Hamilton & Hoise (1970) observed that the uptake of NA by the vascular smooth muscle of the spleen was abolished at 1°C and the NA accumulated in this tissue at 38° could be washed out. A possible reason for the discrepancy between the present results and those of Burnstock *et al.* (1971) may be that they used a much higher concentration of NA (100 μg/ml) and a longer incubation time (60 minutes).

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British Journal of Pharmacology

VOLUME 53 NUMBER 1 JANUARY 1975

page

Systematic Pharmacology

3 MURNAGHAN, M.F. The effect of sympathomimetic amines on the ventricular fibrillation threshold in the rabbit isolated heart.

11 GRAYSON, J. & SCOTT, C. The action of nitroglycerine and dipyridamole in normal and ischaemic dog heart.

21 METCALF, G. & THOMPSON, J.W. The effect of various amine-depleting drugs on the fever response exhibited by rabbits to bacterial or leucocyte pyrogen.

29 BEVAN, P., BRADSHAW, C.M. & SZABADI, E. The effect of tricyclic antidepressants on cholinergic responses of single cortical neurones.

37 MARLEY, E. & WHELAN, JENNIFER E. Some central effects of 5-hydroxytryptamine in young chickens at and below thermoneutrality.

Autopharmacology

43 ADLER-GRASCHINSKY, EDDA & LANGER, S.Z. Possible role of a β-adrenoceptor in the regulation of noradrenaline release by nerve stimulation through a positive feed-back mechanism.

51 KRAFT, E. & ZIMMERMAN, B.G. Influence of histamine H_1- and H_2-receptor blockers on sympathetic vasodilator and vasoconstrictor responses in canine paw.

59 CROCKER, ANN D. & WILSON, K.A. A further investigation into the energy dependence of angiotensin II-induced contractions of isolated smooth muscle preparations.

67 JOHNS, E.J., RICHARDS, H.K. & SINGER, BERTHA. Effects of adrenaline, noradrenaline, isoprenaline and salbutamol on the production and release of renin by isolated renal cortical cells of the cat.

75 LEME, J. GARCIA & SCHAPOVAL, ELFRIDES E.S. Stimulation of the hypothalamo–pituitary–adrenal axis by compounds formed in inflamed tissue.

Miscellaneous

85 ATKINSON, D.C. & HICKS, R. The possible occurrence of endogenous anti-inflammatory substances in the blood of injured rats.

Pharmacokinetics

93 LOVELESS, A.H. & MAXWELL, D.R. The prevention by sulphydryl compounds of the toxicity in the cat of 2,6-dimethoxyphenol and its morpholinopropionyl ester.

99 LULLMANN, H., PETERS, T. & RAVENS URSULA. Studies on the kinetics of $[^3H]$-ouabain uptake and exchange in the isolated papillary muscle of the guinea-pig.

109 FOSTER, R.W. & O'DONNELL STELLA R. Evidence that adrenergic nerves are responsible for the active uptake of noradrenaline in the guinea-pig isolated trachea.

Drug Mechanisms

113 NORTHOVER, B.J. Effect of anti-inflammatory drugs on the membrane potential of vascular endothelial cells *in vitro*.

121 BOULLIN, D.J., GRAHAME-SMITH, D.G., GRIMES, R.P.J. & WOODS, H.F. Inhibition of 5-hydroxytryptamine-induced human blood platelet aggregation by chlorpromazine and its metabolites.

127 HÅKANSON, R., HEDENBRO, J., LIEDBERG, G., REHFELD, J.F. & STADIL, F. Activation of histidine decarboxylase by H_2-receptor blockade: mechanism of action.

131 KOSTERLITZ, H.W. & WATERFIELD, ANGELA A. An analysis of the phenomenon of acute tolerance to morphine in the guinea-pig isolated ileum.

Pharmacometrics

139 BARLOW, R.B. Use of the logistic function for the calculation of dose-ratios and potency ratios.

143 PEARSON, J.D.M. & SHARMAN, D.F. The estimation of 3,4-dihydroxyphenylacetic acid, homovanillic acid and homo-isovanillic acid in nervous tissue by gas–liquid chromatography and electron capture detection.

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page

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157 KELLY, J.G. & SHANKS, R.G. Metabolic and cardiovascular effects of isoprenaline and salbutamol in the dog (SP1, SP3)
163 PAOLONI, H.J. & WILCKEN, D.E.L. The action of aminophylline on the acutely transplanted dog heart: effect of α- and β-adrenoceptor blockade (SP1, DM2)
173 DALY, M.J., FLOOK, JUDITH J. & LEVY, G.P. The selectivity of β-adrenoceptor antagonists on cardiovascular and bronchodilator responses to isoprenaline in the anaesthetized dog (SP1, DM2)
183 McCALDEN, T.A. The inhibitory action of oestradiol-17-β and progesterone on venous smooth muscle (SP1, AP3)
193 MARLEY, E. & NISTICÒ, G. Tryptamines and some other substances affecting waking and sleep in fowls (SP2)

Autopharmacology

207 HAYASHI, E. & YAMADA, S. Pharmacological studies on surugatoxin, the toxic principle from Japanese ivory mollusc (*Babylonia japonica*) (AP1)
217 MAIN, I.H.M. & WHITTLE, B.J.R. Investigation of the vasodilator and antisecretory role of prostaglandins in the rat gastric mucosa by use of non-steroidal anti-inflammatory drugs (AP2)
225 DONOVAN, B.T. Indomethacin, ketoprofen and corpus luteum regression in the guinea-pig (AP2, DM2)
229 NUSTAD, K., PIERCE, J.V. & VAAJE, KIRSTEN. Synthesis of kallikreins by rat kidney slices (AP2, PK2)

Pharmacokinetics

235 BOND, P.A., BROOKS, B.A. & JUDD, A. The distribution of lithium, sodium and magnesium in rat brain and plasma after various periods of administration of lithium in the diet (PK1, SP2)
241 PARVEZ, SIMONE, PARVEZ, S.H. & YOUDIM, M.B.H. Variation in activity of monoamine metabolizing enzymes in rat liver during pregnancy (PK2, AP1, AP3)
247 GARCÍA, A.G. & SÁNCHEZ-GARCÍA, P. Influence of cocaine and sodium on bretylium uptake by reserpine-treated guinea-pig left atrium (PK3, PK2)
257 DA PRADA, M., OBRIST, R. & PLETSCHER, A. Discrimination of monoamine uptake by membranes of adrenal chromaffin granules (PK3, PK1)

Drug Mechanisms

267 KALSNER, S. Role of extraneuronal mechanisms in the termination of contractile responses to amines in vascular tissue (DM1, PK3, AP1)

279 CHIOU, C.Y. & MALAGODI, MARJORIE H. Studies on the mechanism of action of a new Ca^{2+} antagonist, 8-(*N,N*-diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride in smooth and skeletal muscles (DM1)

287 GREEN, A.R., SOURKES, T.L. & YOUNG, S.N. Liver and brain tryptophan metabolism following hydrocortisone administration to rats and gerbils (DM1, SP2)

293 HARRIS, D.W., SMY, J.R., REED, J.D. & VENABLES, C.W. The effects of burimamide and metiamide on basal gastric function in the cat (DM2, SP3, AP2)

Psychopharmacology

299 HOLMAN, R.B., HOYLAND, VALERIE & SHILLITO, ELIZABETH E. The failure of *p*-chlorophenylalanine to affect voluntary alcohol consumption in rats (PP, SP2)

SHORT COMMUNICATIONS

305 GREENWOOD, B. & KERRY, P.J. Prostaglandin production by a mild inflammatory lesion in sheep (AP2)

308 ADAMS, P.R. Kinetics of agonist conductance changes during hyperpolarization at frog endplates (DM1, DM2)

311 SCHILD, H.O. An ambiguity in receptor theory (DM2)

Systematic Pharmacology

SP1	Cardiovascular system
SP2	Central nervous system
SP3	Other physiological systems

Pharmacokinetics

PK1	Storage, distribution, fate
PK2	Synthesis, metabolism
PK3	Uptake, release

Autopharmacology

AP1	Adrenergic, cholinergic transmission
AP2	Histamine, kinins, prostaglandins, etc.
AP3	Hormones

Drug Mechanisms

DM1	Molecular, cellular, biochemical, biophysical
DM2	Receptors, antagonism, potentiation, tolerance

CP	**Clinical Pharmacology, Experimental Therapy**
CT	**Chemotherapy**
M	**Miscellaneous**
PM	**Pharmacometrics**
PP	**Psychopharmacology**
TX	**Toxicology**

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METABOLIC AND CARDIOVASCULAR EFFECTS OF ISOPRENALINE AND SALBUTAMOL IN THE DOG

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1 The intravenous infusion of isoprenaline and salbutamol into the greyhound increased heart rate and levels of free fatty acids, lactic acid and glucose.

2 On terminating the infusions of isoprenaline the changes produced declined rapidly but the effects produced by salbutamol were more persistent. When high doses of salbutamol had been infused, glucose and lactic acid levels in fact increased during the 20 min following the infusions.

3 These results support suggestions that, in the dog, lipolysis is mediated by β_1-adrenoceptors and liver glycogenolysis by β_2-adrenoceptors. The β-adrenoceptors mediating muscle glycogenolysis could not be assigned unequivocally to either subtype.

4 The differences in the behaviour of isoprenaline and salbutamol in the period following the infusions are considered to be due partly to slower removal of salbutamol. Increases in lactic acid levels after infusion of large amounts of salbutamol may be secondary to the persistence of high glucose levels.

Introduction

Previous studies indicate that liver and muscle glycogenolysis in the dog are mediated by β-adrenoceptors. McCutcheon (1962) showed that the β-adrenoceptor blocking agent, dichloroisoprenaline (DCI), inhibited catecholamine-induced rises in lactic acid and glucose levels in the dog and that the α-adrenoceptor blocking agent, phenoxybenzamine, had no effect on these responses. Mayer, Moran & Fain (1961) demonstrated that DCI inhibited the rises in fatty acid, lactic acid and glucose levels produced by infusions of adrenaline in the dog. When they proposed the subdivision of β-adrenoceptors, Lands, Arnold, McAuliff, Luduena & Brown (1967) suggested that lipolysis and cardiac stimulation were mediated by β_1-adrenoceptors and that bronchodilatation and vasodepression were mediated by β_2-adrenoceptors. Arnold, McAuliff, Colella, O'Connor & Brown (1968) examined the relative effects of isoetharine, adrenaline and noradrenaline in producing hyperglycaemia and hyperlacticacidaemia in the dog and suggested that both effects were mediated by β_2-adrenoceptors, since isoetharine was more potent than adrenaline or noradrenaline in producing them.

Salbutamol, a drug which has a greater effect on β_2- than on β_1-adrenoceptors, has been studied by Cullum, Farmer, Jack & Levy (1969) and by Ekue, Shanks & Zaidi (1971). These workers showed that in dogs, salbutamol had approximately one-eighth the potency of isoprenaline in producing increases in femoral blood flow (a β_2-adrenoceptor response) but had only one-two hundredth the potency of isoprenaline in increasing heart rate (a β_1-adrenoceptor response) but no study of the metabolic effects of this compound in the dog has been reported. Such a study is described here. The examination of salbutamol in comparison with isoprenaline was expected to elucidate further the division of the β-adrenoceptors involved in metabolic responses into β_1- and β_2-subtypes. Changes in heart rate and femoral blood flow produced by isoprenaline and salbutamol have been measured in parallel.

Methods

Observations were made in greyhounds of either sex, weighing 17-30 kg. The animals were fasted for 24 h prior to experiments. The dogs were anaesthetized with pentobarbitone (30 mg/kg i.v.) and artificially respired with room air using a Starling Ideal pump at a rate of 18 strokes/min and a stroke volume of 13 ml/kg body weight. Needle electrodes were inserted subcutaneously for recording the electrocardiogram (Lead II) and heart rate.

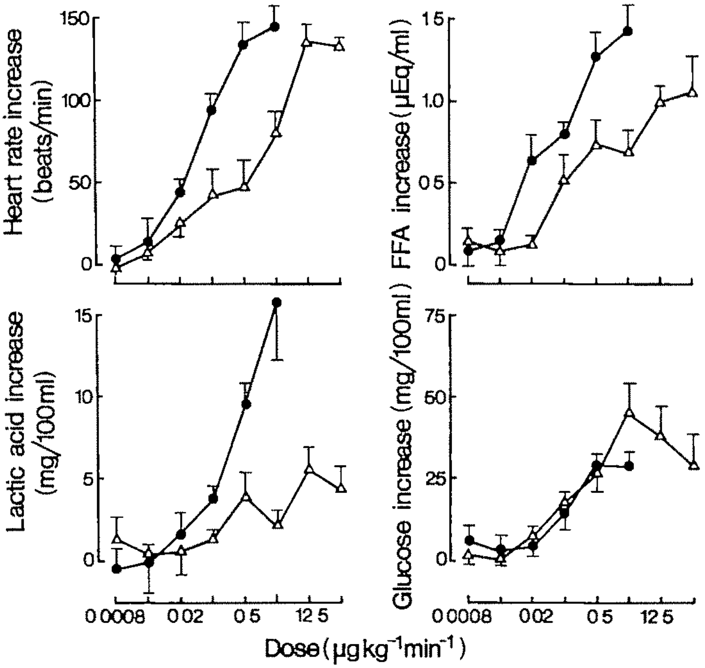

Figure 1 Mean increases in heart rate and in free fatty acids (FFA), lactic acid and glucose levels at the end of the intravenous infusion for 10 min of a series of doses of isoprenaline (●) and salbutamol (△). Each point for isoprenaline is the mean of observations in six dogs and each point for salbutamol is the mean of observations in five dogs. Vertical bars show s.e. mean.

Intravenous infusion experiments

Polyethylene catheters were inserted into each saphenous vein, one for the administration of drugs by constant intravenous infusion and the other for obtaining blood samples for the measurement of free fatty acids, lactic acid and glucose levels. Isoprenaline and salbutamol were given as constant intravenous infusions for 10 min at the following rates: isoprenaline 0.0008, 0.004, 0.02, 0.1, 0.5, 2.5 μg kg^{-1} min^{-1} and salbutamol 0.0008, 0.004, 0.02, 0.1, 0.5, 2.5, 12.5, 62.5 μg kg^{-1} min^{-1}. Concentrations of each drug in 0.9% w/v NaCl solution (saline) were adjusted so that the required dose was given in a volume of 2 ml per minute. Blood samples were taken before and at the end of each infusion and again 20 min later. Isoprenaline infusions were repeated after 90 min when all parameters measured had returned to baseline values; three doses of isoprenaline were infused in randomized order into each dog. Isoprenaline was given to a total of 12 dogs. Since the effects of salbutamol were prolonged, two doses were given to each dog with 2 h between each infusion. Salbutamol was given to a total of 20 dogs. As the effects of salbutamol, 12.5 μg kg^{-1} min^{-1} and 62.5 μg kg^{-1} min^{-1} were very prolonged (>6 h), neither dose was administered as the first dose in an experiment.

Observations were made in four dogs in which glucose was infused intravenously at 20 mg kg^{-1} min^{-1} for 20 min (in 2 ml water/minute). Blood samples were taken for estimation of glucose and lactic acid levels before an infusion and every 10 min for the next 40 minutes.

Intra-arterial infusions in the hind limb

Changes in uptake and release of free fatty acids, glucose and lactic acid in response to the administration of isoprenaline and salbutamol were estimated by the Fick principle. An external iliac artery was exposed through a lower abdominal incision and the probe of an electromagnetic flowmeter (Medicon, K 2000) was applied to the artery proximal to the origin of the deep femoral branch. Mean blood flow was recorded. A polyethylene catheter was inserted into the deep femoral artery until its tip lay in the main artery distal to the flow probe. This catheter allowed administration of drugs and sampling of blood entering the hind limb. A second catheter

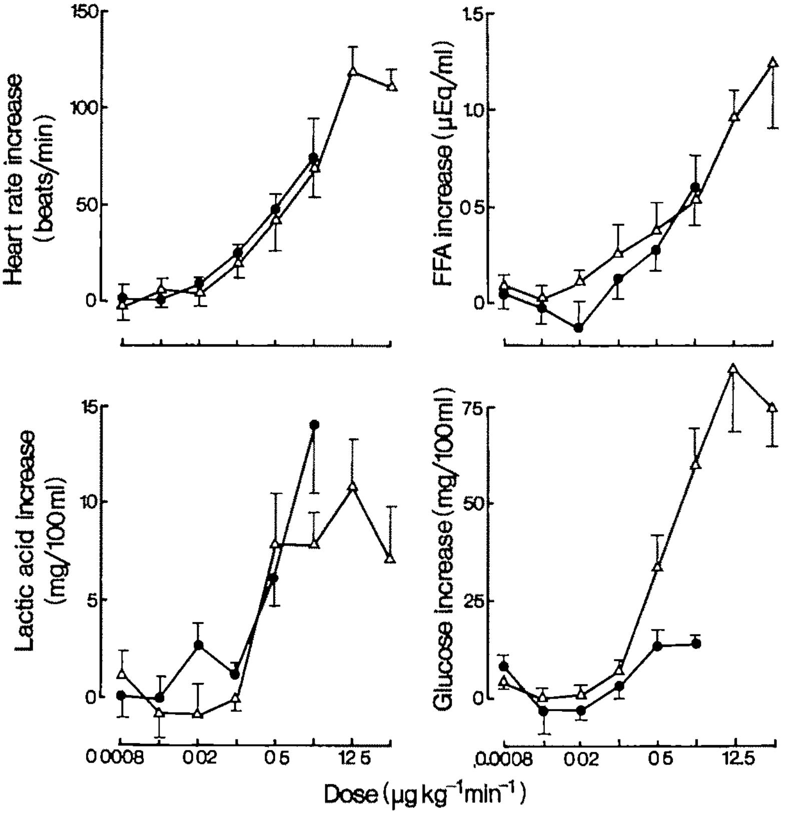

Figure 2 Mean increases in heart rate and in free fatty acids (FFA), lactic acid and glucose levels obtained 20 min after the intravenous infusion of a series of doses of isoprenaline (●) and salbutamol (△). Each point for isoprenaline is the mean of observations in six dogs and each point for salbutamol is the mean of observations in five dogs. Vertical bars show s.e. mean.

was inserted into a branch of the femoral vein to allow sampling of blood leaving the hind limb. Blood was taken from both sampling sites for estimation of free fatty acids, lactic acid and glucose before an infusion of isoprenaline or salbutamol and at the end of each infusion. Isoprenaline and salbutamol were given as 10 min infusions into the femoral artery at the following rates: isoprenaline 0.002, 0.006, 0.018 µg kg^{-1} min^{-1} and salbutamol 0.006, 0.018, 0.054 µg kg^{-1} min^{-1}. Drugs were dissolved in saline and the concentrations were adjusted so that the required dose was given in 0.5 ml per minute.

Isoprenaline infusions were repeated after 90 min and three infusions were carried out on one day. Salbutamol infusions were repeated after 2 h and only two infusions were given to each dog. Isoprenaline was administered to five dogs and salbutamol was administered to six dogs.

Plasma concentrations of free fatty acids were estimated by the method of Mosinger (1965). Lactic acid in blood was determined by the enzymatic method of Hohorst, Krentz & Bucher (1959) and blood glucose by the method of Hultman (1959).

Drugs used were: (±) isoprenaline sulphate (Burroughs Wellcome) and (±)-salbutamol sulphate (Allen and Hanburys). All doses are expressed in terms of the base.

Results

Intravenous infusion of drugs

Mean increases in heart rate, free fatty acids, lactic acid and glucose produced by a series of increasing doses of isoprenaline in six dogs, and salbutamol in five dogs, are shown in Figure 1 in which the values obtained at the end of the period of drug administration are given.

Maximum increases in heart rate produced by isoprenaline and salbutamol were not significantly different from each other. The upper part of the log dose-response curve for salbutamol was parallel to and to the right of that for isoprenaline but the lower part of the salbutamol curve was flatter than that for isoprenaline. Doses causing 50% of the maximum response were on the parallel parts of the curves, and their comparison showed salbutamol to have one-thirtieth the potency of isoprenaline on a weight basis.

The log dose-response curve for the effect of salbutamol on free fatty acid levels was situated to the right of that for isoprenaline and there was no significant difference between the maximum increases produced by the two drugs. The increase produced by the intravenous infusion of isoprenaline at 0.5 μg kg^{-1} min^{-1} was significantly greater ($P < 0.05$) than that produced by the same dose of salbutamol. Salbutamol produced its maximum lipolytic effects at 12.5 μg kg^{-1} min^{-1}.

The log dose-response curves for isoprenaline and salbutamol on glucose levels overlapped, no differences between the responses to the same doses of the two drugs being significant, but isoprenaline produced its maximum effects at 0.5 μg kg^{-1} min^{-1} and salbutamol at 2.5 μg kg^{-1} min^{-1}.

Isoprenaline produced dose-related increases in lactic acid levels at doses up to 2.5 μg kg^{-1} min^{-1}. Salbutamol had little effect on lactic acid levels, a significant increase ($P < 0.05$) being produced only by 12.5 μg kg^{-1} min^{-1}. Therefore a potency ratio could not be calculated.

As compared with the values found at the end of the infusion of isoprenaline (Figure 1), the values found 20 min later (Figure 2) had declined markedly in the case of heart rate, free fatty acids and glucose (all values) and slightly in the case of lactic acid ($P < 0.05$ for 0.1 and 0.5 μg kg^{-1} min^{-1} values). In contrast, at 20 min after termination of the salbutamol infusions, heart rate and free fatty acid increases were maintained. Moreover, after the higher doses of salbutamol the levels of glucose and lactic acid rose further during the 20 min period after the end of the infusion. At this time salbutamol and isoprenaline were about equipotent in their effects on heart rate, free fatty acid and lactic acid levels. The effect on glucose of salbutamol was much greater than that of isoprenaline, the slopes of the two dose-response curves differed.

Intravenous administration of glucose

Figure 3 shows the mean results of four experiments in which glucose was administered to anaesthetized dogs as an intravenous infusion at 20 mg kg^{-1} min^{-1} for 20 minutes. The blood glucose levels increased to values comparable to those found after administration of salbutamol and fell rapidly after the infusion was stopped. The lactic acid levels rose significantly ($P < 0.05$) during the infusion of glucose and were maintained during the following 20 minutes.

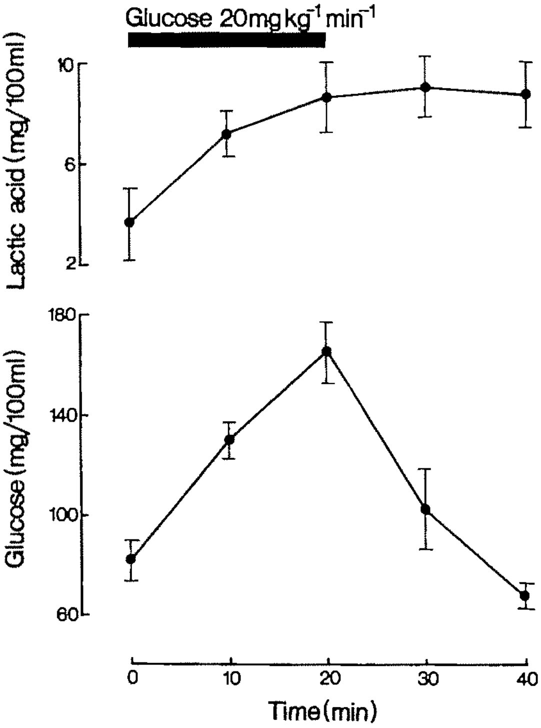

Figure 3 Mean values for lactic acid and glucose in four anaesthetized dogs in which glucose was given by intravenous infusion at 20 mg kg^{-1} min^{-1} during the period indicated. Vertical bars show s.e. mean.

Intra-arterial infusion in the hind limb

During the infusion of isoprenaline and of salbutamol into the femoral artery in anaesthetized dogs, heart rate tended to increase (not significant) and the concentrations in femoral arterial blood of free fatty acids, lactic acid and glucose did not change. Changes in femoral blood flow and in uptake or output of lactic acid found in the hind limb just after intra-arterial infusions of increasing doses of isoprenaline and salbutamol are shown in Figure 4. Isoprenaline and salbutamol had no significant effect on glucose or free fatty acid metabolism. Salbutamol did not change lactic acid levels but isoprenaline produced a dose-related increase in the output of lactic acid by the limb. Both isoprenaline and salbutamol increased blood flow to the hind limb and the salbutamol

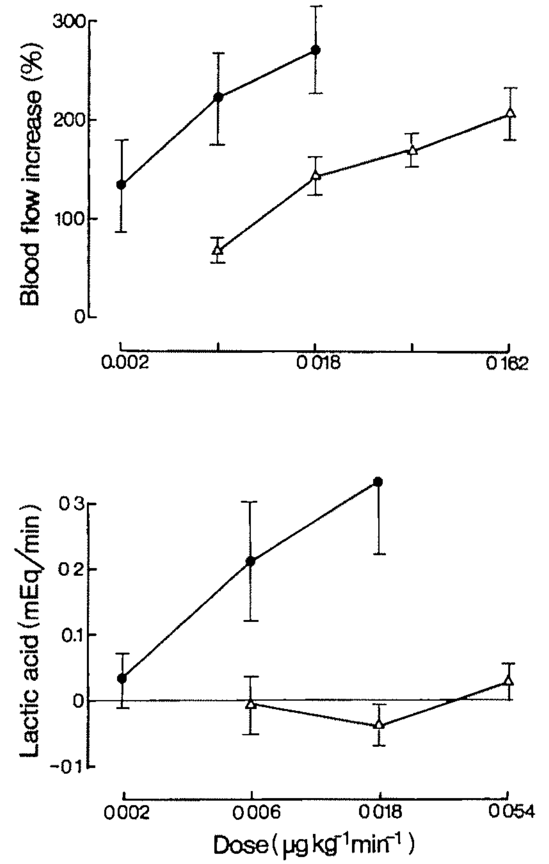

Figure 4 Mean changes in blood flow to the hind limb and output (+) or uptake (—) of lactic acid in the hind limb of anaesthetized dogs produced by the intra-arterial infusion for 10 min of a series of doses of isoprenaline (●) and salbutamol (△). Each point is the mean of five observations for isoprenaline and of four observations for salbutamol. Vertical lines show s.e. mean.

dose-response curve was to the right of the isoprenaline curve.

Discussion

The intravenous infusion of isoprenaline and salbutamol increased heart rate. Although the two log-dose response curves were of different shapes, the upper parts of each were parallel and over these dose ranges isoprenaline was 30 times more active than salbutamol. In previous studies by Ekue *et al.* (1971) in anaesthetized dogs, isoprenaline was 200 times more active than salbutamol in increasing heart rate, but in this study full dose-response curves were not examined and the drugs were administered as single intravenous doses instead of by infusion. Neither study attempted to assess the influence of reflex vagal effects produced by the drugs.

The values obtained at the termination of their intravenous infusions indicate that salbutamol is equiactive with isoprenaline in increasing blood glucose but less active in increasing heart rate, free fatty acids and lactic acid. As previous observations (Cullum *et al.*, 1969, Ekue *et al.*, 1971) have indicated that salbutamol has a greater effect on β_2- than on β_1-adrenoceptors and as the heart rate response is classified as a β_1 effect (Lands *et al.*, 1967), the present results suggest that in the dog the increase in blood glucose produced by isoprenaline and salbutamol is mediated by β_2-adrenoceptors and that the free fatty acid response is mediated through β_1-adrenoceptors. Such suggestions agree well with those of Lands *et al.* (1967) and Arnold *et al.* (1968) who suggested that in the rat, catecholamine-induced lipolysis like tachycardia was due to stimulation of β_1-adrenoceptors and that in the dog liver glycogenolysis was mediated by β_2-adrenoceptors.

Isoprenaline by close arterial infusion was more active than salbutamol in increasing blood flow to the hind leg and isoprenaline increased the output of lactic acid whereas salbutamol did not. This observation, together with the finding that salbutamol produced minimal increases in lactic acid levels at the end of intravenous infusions, indicates that muscle glycogenolysis may be mediated by β_1-adrenoceptors. In contrast Arnold *et al.* (1968) suggested that muscle glycogenolysis in the dog is mediated by β_2-adrenoceptors following studies of the maximum increases in lactic acid produced in anaesthetized dogs by the intravenous infusion of isoprenaline, noradrenaline and isoetharine.

The increased levels of heart rate, free fatty acids, glucose and lactic acid present at the end of the infusion of isoprenaline declined during the 20 min period which followed. In contrast, in this period after infusion of salbutamol, the heart rate and free fatty acid responses did not fall and when the dose was high, glucose and lactic acid levels increased. This may result at least in part from the longer half-life of salbutamol, and a delayed onset of peak metabolic change. The delayed increases in lactic acid levels after salbutamol may partially result from a conversion of glucose to lactic acid in the manner suggested by Forbath & Hetenyi (1970). That such a conversion could have occurred was shown by the increase in lactic acid produced by the infusion of glucose. The lactic acid response to isoprenaline fell much more slowly than any of the other responses to this drug and this may also have arisen from conversion of glucose to lactic acid.

As well as comparing the effects of isoprenaline and salbutamol on some metabolic processes, this study also illustrates some difficulties encountered when examining *in vivo* subdivisions of adrenoceptors mediating metabolic responses. Difficulties occur because drugs vary in persistence and are not completely selective for one receptor subtype and because of the physiological interrelationships existing between some responses (Fleming & Kenny, 1964; Antonis, Clark, Hodge, Molony & Pilkington, 1967).

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THE ACTION OF AMINOPHYLLINE ON THE ACUTELY TRANSPLANTED DOG HEART: EFFECT OF α- AND β-ADRENOCEPTOR BLOCKADE

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1 Aminophylline inhibits the coronary vasodilator actions of adenosine. Our previous studies suggested that low dose infusions of aminophylline reduce coronary blood flow in the isolated heart. In the present study we investigated the actions of aminophylline on coronary blood flow and myocardial contractility in a transplanted heart model. Drugs were given by close coronary arterial infusion.

2 Aminophylline in low doses (200 μg/min) reduced coronary blood flow by 21 ± 2% (mean ± s.e. mean) but did not alter myocardial contractility or heart rate. Higher doses (500 and 1000 μg/min) increased coronary blood flow and myocardial contractility without changing heart rate.

3 α-Adrenoceptor blockade with phenoxybenzamine did not affect the response to a low dose of aminophylline (200 μg/min).

4 Propranolol in doses of 10 and 30 μg/min blocked β-adrenoceptors but did not change coronary blood flow. The higher dose reduced myocardial contractility.

5 The effects of a high dose of aminophylline (1000 μg/min) on coronary blood flow were not changed by either α- or β-adrenoceptor blockade, although propranolol (30 μg/min) reduced the augmentation in myocardial contractility.

6 The results show that when given in doses which do not alter myocardial contractility, aminophylline reduces coronary blood flow in the isolated heart and that this is not mediated through an α-adrenoceptor mechanism, They also show that the increases in coronary blood flow and positive inotropic effects obtained with higher doses of aminophylline are not mediated through catecholamines and suggest that higher doses of aminophylline have a small direct coronary vasodilator action. The low dose vasoconstrictor response may be produced by inhibition of the coronary vasodilator action of locally produced adenosine.

Introduction

Aminophylline is widely used in the treatment of bronchial airways obstruction and cardiac failure. The active component theophylline, in combination with ethylenediamine to enhance solubility, produces therapeutic effects largely through its actions on smooth muscle and cardiac muscle (Goodman & Gilman, 1970).

The mechanisms are only partly understood and are controversial (Epstein, Levey & Skelton, 1971). Aminophylline has been reported to have a coronary vasodilator action in the intact heart of both man (Starr, Gamble, Margioles, Donal, Joseph & Eagle, 1937; Howarth, McMichael & Sharpey-Schafer, 1947; Charlier, 1961) and the dog (Stoland, Ginsberg, Loy & Hiebert, 1934; Boyer & Green, 1941; Boyer, 1943) and to increase the energy requirements and contractile state of the heart (Melville & Lu, 1950; Amsterdam, Zelis, Spann, Miller & Mason, 1970). There is evidence that aminophylline influences calcium exchange in the heart (Nayler, 1963; Nayler & Hasker, 1966; Nayler, 1967; McNeill, Nassar & Brody, 1969; Blinks, Olson, Jewell & Braveny, 1972; Massingham & Nasmyth, 1972), that it increases intracellular cyclic adenosine-3,5'-monophosphate (cyclic AMP) through inhibition of phosphodiesterase (Butcher & Sutherland, 1962; Sutherland, Robison & Butcher, 1968) and that it releases catecholamines in the heart (Westfall & Fleming, 1968; Marcus, Skelton, Grauer & Epstein, 1972) and potentiates their effects on blood vessels (Rall & West, 1963; Bartelstone, Nasymth & Telford, 1967; Massingham, 1969; Kalsner, 1971). An additional direct effect has also been proposed (Marcus *et al.*, 1972; Cohen, Lesne, Valette & Wepierre, 1970).

Our interest in aminophylline resulted from the observation in the transplanted heart that low dose infusions of aminophylline reduced coronary blood flow and increased coronary vascular resistance (Paoloni & Wilcken, 1971, 1972). The aminophylline had been given as part of a study of its inhibition of the coronary vasodilator action of adenosine and other coronary vasodilators. The present study was undertaken to determine the effect of aminophylline on both the coronary blood flow and the contractile state of the transplanted heart and to investigate the role of catecholamines in the aminophylline response by the use of selective adrenoceptor blockade.

Methods

Experimental model

A dog heart (the donor heart) was transplanted into the neck of a second dog (the recipient dog) as described previously (Paoloni & Wilcken, 1971, 1972). The donor heart (weight 90-130 g) was transplanted into the neck of the recipient dog by anastomosing the subclavian artery to the proximal end of the recipient's carotid artery whilst the donor's left pulmonary artery was anastomosed to the recipient's external jugular vein. The donor heart continued to beat forcefully in sinus rhythm throughout the procedure. In this preparation, blood flows into the donor aorta from the recipient's carotid artery at a pressure equal to the recipient dog's systemic pressure. As the superior and inferior venae cavae and azygous veins of the donor heart are ligated, the coronary venous blood of the donor heart is returned to the recipient's circulation through the left pulmonary artery to the external jugular vein anastomosis.

The coronary blood flow (CBF) in this preparation is the volume of blood per unit time which enters the donor aorta from the recipient's carotid artery, provided that the aortic valve is competent (Paoloni & Wilcken, 1971). To measure CBF, an electromagnetic flow probe (IVM Model FT-2HS) was placed on the feeding carotid artery proximal to the anastomosis and connected to an EMI Electromagnetic Flowmeter (Type 28) as previously described (Paoloni & Wilcken, 1971). The perfusion pressure in the donor aorta was measured through a triple lumen catheter (Polyvinyl o.d. 2.50-i.d. 0.96 mm each) inserted through the innominate artery, positioned above the aortic valve and connected to a Statham Pressure Transducer (Model P23.DB). This catheter was also used for drug infusions. The donor electrocardiogram (ECG) was recorded from myocardial electrodes attached to the left ventricle.

The donor left ventricular pressure was measured from a 5 cm long stiff-walled catheter (Bardic Polyvinyl Chloride Angiocath 14 Gauge, o.d. 2.00 mm-i.d. 1.5 mm) introduced transmurally into the left ventricular cavity through the apex. This catheter was connected directly to a Statham P23.DB Pressure Transducer. The dynamic response of the complete catheter/transducer system was checked with a sine wave pressure generator (Gersh, Hahn & Prys-Roberts, 1971; Nejad, Klein, Mirsky & Lown, 1971). When tested in this manner the system had an amplitude/frequency response flat (±5%) to 35 Hz. The ECG and pressure and flow signals were displayed on an oscilloscope screen and recorded on photographic paper with an Electronics for Medicine DR8 Multichannel Recorder. The left ventricular pressure and its electronically derived first derivative (dP/dt) were recorded at a paper speed of 200 mm/s for accuracy of measurement. The dP/dt was measured directly from the left ventricular pressure trace, the electronically derived first derivative (dP/dt) being used only as a guide to the point in the trace at which peak dP/dt occurred. The left ventricular Isometric Tension Time Index (dP/dt^{-1} IIT^{-1}) (Siegel & Sonnenblick, 1963; Siegel, Sonnenblick, Judge & Wilson, 1964; Siegel, 1969) was calculated by integrating (by planimetry) the left ventricular pressure trace from the end-diastolic point to the point of peak dP/dt to obtain the Integrated systolic Isometric Tension (IIT) value and dividing the measured peak dP/dt by IIT. Thus developed pressure was used in the calculation of IIT, not total pressure.

The CBF was measured as the area (by planimetry) under the flow signal per unit of time and converted to ml per min from the direct flow measurement and the calibration signal (Paoloni & Wilcken, 1971). The coronary vascular resistance was estimated as mean donor aortic pressure/CBF. The right atrium of the donor heart was observed to be collapsed during the studies and right atrial pressure was therefore not measured.

There were 20 successful preparations; studies were not performed on any preparation displaying cardiac irregularities or left ventricular distension. The preparations remained stable as regards perfusion pressure, coronary blood flow and arterial blood gases and pH for at least 6 h (Paoloni & Wilcken, 1971).

When control tracings taken between 30 and 60 min after transplantation were found to be steady, drug studies were begun. To avoid the effects of any transient minor changes in CBF each drug-induced alteration in CBF was compared

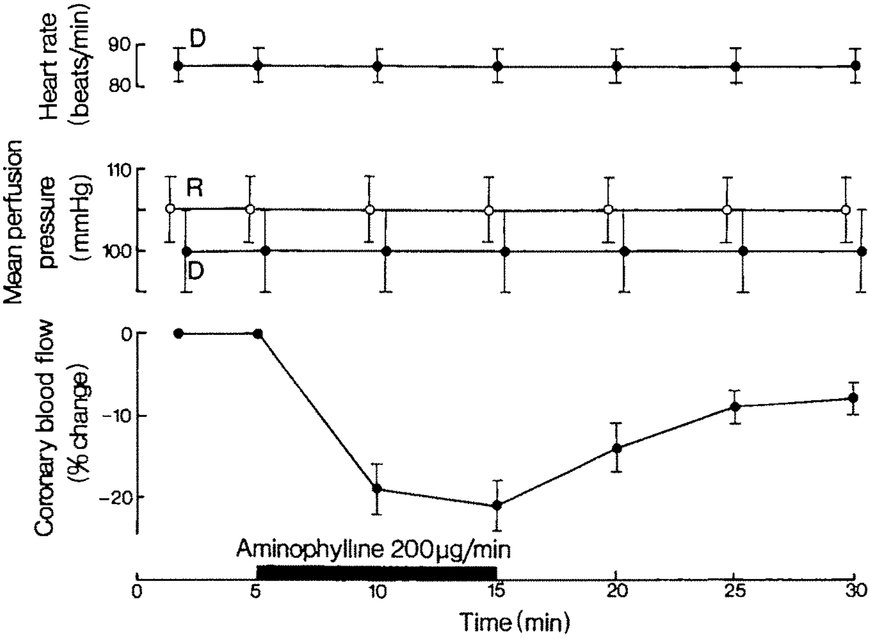

Figure 1 Grouped data from 12 experiments in 12 dogs, showing the changes in coronary blood flow (mean ± s.e. mean) produced by 10 min infusions of aminophylline 200 µg/minute. There were no changes in donor heart rate (D) or in recipient (R) and donor (D) mean aortic pressures.

to a 1 min control flow immediately preceding the drug study. At the end of the studies, each heart remained in sinus rhythm without ectopic beats. There was no cardiac distension.

Drug studies

The drugs in 0.9% w/v NaCl solution (saline) were infused at 1 ml/min (Braun Infusion Pump) into the aorta of the donor heart via the triple lumen catheter whose tip was above the aortic valve. Allowance was made for a catheter transit time of 30 seconds.

In the analysis of the results, Student's *t* test was used to determine the significance of differences between paired observations in each series of experiments.

Results

In all preparations the CBF was found to be steady at 30 to 60 min after transplantation. At 60 min mean CBF (±s.e. mean) was 81 ± 4 ml min^{-1} 100 g^{-1} (range 66 to 106 ml). At the conclusion of each 4 to 6 h study, resting flow had not decreased by more than 10%.

Aminophylline

In each experiment an infusion of aminophylline 200 µg/min into the donor heart reduced CBF. The decrease began within 1 to 2 min of starting the infusion and CBF was at its lowest value after 5 minutes. The grouped responses obtained from 12 experiments in 12 dogs are shown in Figure 1. The CBF was reduced by 19.3 ± 2.5% at 5 min and 21.5 ± 2.7% at 10 min without any change in donor heart rate or perfusion pressure ($P > 0.05$) and no significant alteration in the indices of ventricular contractility (dP/dt, $dP\ dt^{-1}\ IIT^{-1}$) ($P > 0{,}05$) (Figure 2). On stopping the infusion at 10 min the CBF returned towards, but did not invariably reach, pre-infusion control values (Figure 1). There was, however, a significant increase in CBF 10 min after stopping the infusion ($P < 0.0005$).

In order to determine the influence of ethylenediamine in the low dose aminophylline response, an infusion of ethylenediamine 30 µg/min was given separately, this representing the proportion of ethylenediamine present in the aminophylline 200 µg/min infusion. In 5 experiments in 5 preparations, a 5 min infusion of ethylenediamine did not significantly alter CBF or

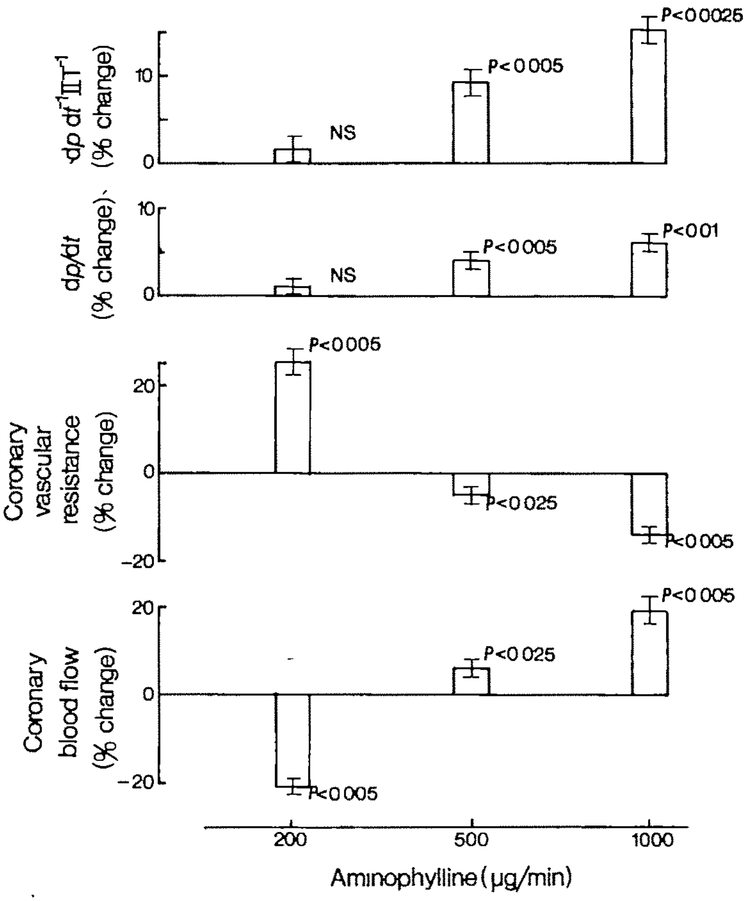

Figure 2 The changes (group data, mean ± s.e. mean) in coronary blood flow, coronary vascular resistance and myocardial contractility ($dP/dt\ dP\ dt^{-1}\ \mathrm{IIT}^{-1}$) produced by 10 min infusions of aminophylline 200 μg/min (10 experiments), 500 μg/min (6 experiments) and 1000 μg/min (6 experiments). There were no significant changes in donor heart rate or perfusion pressure.

Table 1 Changes in coronary blood flow produced by 10-min infusions of aminophylline

Aminophylline	n	*Coronary blood flow (ml min^{-1} 100 g^{-1})*		*Coronary blood flow (% change)*	P
		Control	*Response*		
200 μg/min	12	78.6 ± 6.0 (55-107)	62.8 ± 5.5 (35-96)	−21.5 ± 2.7	<0.005
500 μg/min	6	83.4 ± 4.5 (66-106)	88.9 ± 6.0 (68-120)	+6.2 ± 2.0	<0.025
1000 μg/min	6	82.0 ± 6.8 (66-106)	97.8 ± 9.7 (72-130)	+19.4 ± 4.5	<0.005

Measurements are mean with s.e. mean. Figures in parentheses indicate range.

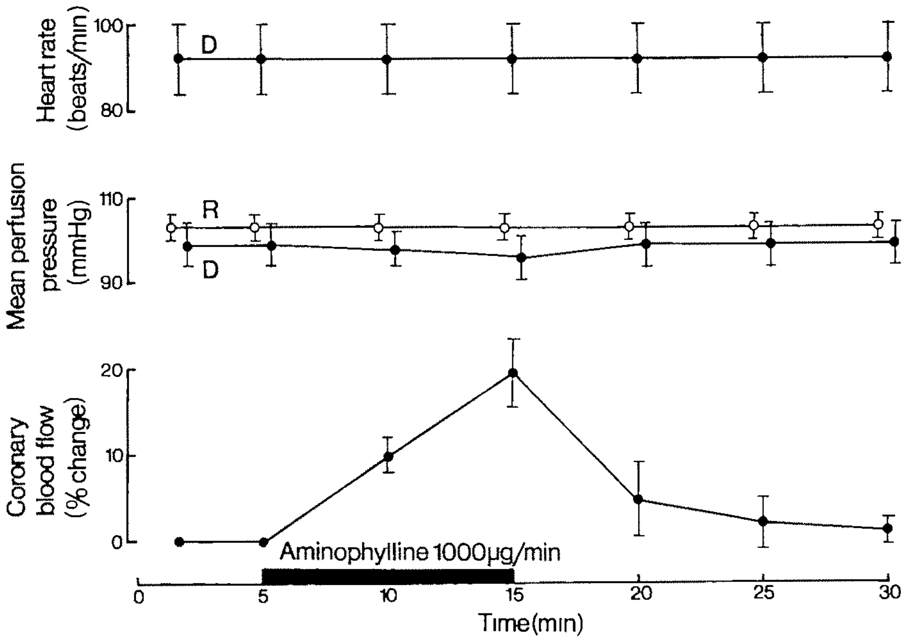

Figure 3 Changes in coronary blood flow (mean ± s.e. mean), recipient (R) and donor (D) mean perfusion pressure and donor heart rate produced by 10 min infusions of aminophylline 1000 µg/min (6 experiments in 6 dogs).

coronary vascular resistance, suggesting that the observed aminophylline responses were due to the theophylline component alone.

A higher dose infusion of aminophylline (1000 µg/min) increased CBF in every experiment. The combined results from 6 experiments in 6 dogs (Figure 3) show an increase in CBF at 5 min of 9.8 ± 2.0% and at 10 min of 19.4 ± 4.5% when the infusion was stopped. The CBF returned to pre-infusional control values by 10 to 15 minutes. There were no significant changes in donor heart rate or perfusion pressures, although both dP/dt and dP dt^{-1} IIT^{-1} were significantly increased ($P < 0.005$) (Figure 2). In a further 6 experiments, a 10 min infusion of aminophylline 500 µg/min produced an increase in CBF of 6.2 ± 2.0% with a significant increase in dP/dt and dP dt^{-1} IIT^{-1} ($P < 0.05$) (Figure 2 and Table 1).

Aminophylline and α-adrenoceptor blockade

The mechanism of the aminophylline-induced decrease in CBF was investigated 60 min after premedication of the recipient dog with phenoxybenzamine (2 mg/kg intravenously). The phenoxybenzamine reduced the recipient dog's mean aortic pressure by 16 ± 3 mmHg (mean ± s.e. mean) over this period. The degree of α-adrenoceptor blockade produced by phenoxybenzamine was assessed by giving rapid intravenous injections of 30 µg of phenylephrine to the recipient dog and recording the increase in the recipient dog's mean aortic pressure. Before phenoxybenzamine the increase was 8.0 ± 0.8 mmHg ($P < 0.01$), whereas after phenoxybenzamine, phenylephrine had no significant effect. In 5 preparations after phenoxybenzamine, aminophylline 200 µg/min decreased CBF to a similar extent as in the control studies (Figure 4). In 2 experiments in 2 dogs, aminophylline 1000 µg/min was infused after phenoxybenzamine. The usual increases in CBF, dP/dt and dP dt^{-1} IIT^{-1} were obtained. In one experiment, the phenoxybenzamine was slowly infused directly into the donor heart. The responses to both aminophylline 200 µg/min and 1000 µg/min were unchanged 1 h after the infusion.

Aminophylline and β-adrenoceptor blockade

The effect of β-adrenoceptor blockade on the aminophylline responses was investigated with propranolol. A 5 min infusion of propranolol 30 µg/min did not significantly change CBF, donor heart rate or perfusion pressure (Paoloni &

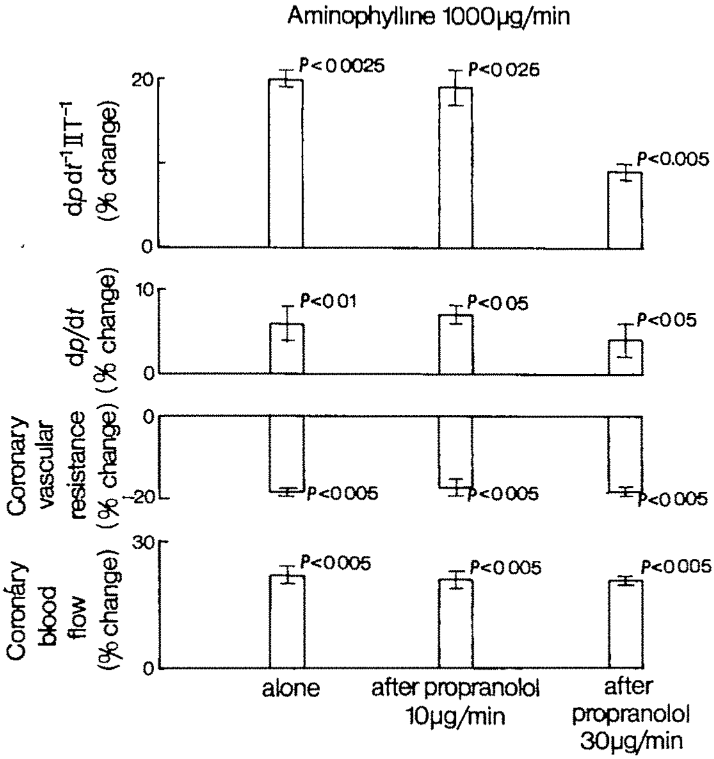

Figure 4 The changes in coronary blood flow (mean ± s.e. mean), coronary vascular resistance and myocardial contractility (dP/dt, $dP\ dt^{-1}\ IIT^{-1}$) produced by 10 min infusions of aminophylline 1000 µg/min, when given alone (10 experiments), after propranolol 10 µg/min (5 experiments) and after propranolol 30 µg/min (5 experiments). The control data comprise controls from both sets of experiments.

Wilcken, 1971), yet fully prevented the increases in CBF and donor heart rate produced by 2 min infusions of isoprenaline 0.5 µg/min given into the donor aorta. This effect was maintained for at least 60 minutes. Propranolol 30 µg/min produced a small negative inotropic effect on the transplanted heart and reduced left ventricular dP/dt and $dP\ dt^{-1}\ IIT^{-1}$ by 4 ± 1 and 13 ± 2% respectively.

When aminophylline 1000 µg/min was infused in 5 preparations after an infusion of propranolol 30 µg/min the increases in CBF were not significantly different from those obtained before β-receptor blockade ($P > 0.05$). However, the contractility changes produced by the high dose of aminophylline were decreased after this dose of propranolol ($P < 0.125$) (Figure 4). On repeating the experiments in 5 dogs with a lower, though still a β-receptor blocking, dose of propranolol (10 µg/min) the aminophylline-induced increases in CBF and contractility were not altered, showing that β-receptor blockade with propranolol did not modify the actions of aminophylline 1000 µg/minute.

To check the sensitivity of the left ventricular dP/dt and $dP\ dt^{-1}\ IIT^{-1}$ measurements as indices of changes in myocardial contractility, the effects of isoprenaline and ouabain on dP/dt and $dP\ dt^{-1}\ IIT^{-1}$ were compared with aminophylline. Infusions of isoprenaline 0.5 µg/min increased dP/dt and $dP\ dt^{-1}\ IIT^{-1}$ by 80 ± 5% and 200 ± 50% (mean ± s.e. mean) respectively. Intravenous injections of 0.75 mg of ouabain given to the recipient dog increased the dP/dt of the donor heart by 9 ± 3% and the $dP\ dt^{-1}\ IIT^{-1}$ by 19 ± 3% within 15 min without altering the donor heart rate. The mean blood pressure of the recipient dog was however increased by 9 ± 1 mmHg following the ouabain injection.

Discussion

With the methods used, we were able to transplant a beating heart which was perfused continuously and was not subjected to an anoxic period. The preparation seemed suitable for the study of the effects of vasoactive drugs on the coronary circulation of the acutely transplanted and denervated heart (Paoloni & Wilcken, 1971; 1972).

The methods used to assess changes in myocardial contractility (dP/dt and $dP\ dt^{-1}\ \mathrm{IIT}^{-1}$) were based on the studies of Siegel & Sonnenblick (1963), Siegel *et al.* (1964), and Siegel (1969), measurements being made during the isometric phase of left ventricular contraction (see methods section). The accuracy is critically dependent on the fidelity of the pressure recording system. The frequency/response characteristics of the left ventricular catheter/transducer system we used were flat (+5%) to 35 Hz. As the resting donor heart rate was always less than 120/min (usually about 90/min) the pressure recordings were of sufficient fidelity to represent accurately the 15th to 20th harmonic of the pressure pulse. This is adequate for the measurement of peak dP/dt (McDonald, 1960; Gersh *et al.*, 1971). In support of this, the indices we used reflected appropriately not only the gross contractility changes which isoprenaline is known to produce but also the smaller changes after ouabain which were of approximately the same order of magnitude as those we found with aminophylline. Further, during the aminophylline studies the donor heart rates, donor aortic pressures and donor left ventricular end-diastolic pressures remained steady so that the left ventricular pre-load and after-load should not have been altered (Grabner, Conti, Lappe & Ross, 1972). Under these conditions the indices derived from the left ventricular pressure trace which we used appeared suitable for the assessment of changes in myocardial contractility produced by infusions of aminophylline.

Our studies showed that aminophylline given in low doses (200 μg/min) which did not alter myocardial contractility or heart rate, reduced CBF and produced coronary vasoconstriction. When high doses of aminophylline (1000 μg/min) were infused, both CBF and myocardial contractility were increased. There were no changes in donor heart rate. With aminophylline doses of 500 μg/min, the changes were similar to those found with 1000 μg/min but were of smaller magnitude. The doses of aminophylline delivered to the coronary circulation of the transplanted heart were of a similar order to those used clinically in the treatment of bronchial airways obstruction or congestive cardiac failure. Assuming that there is a simple dilution of the drug in the circulating blood volume, an intravenous injection of 250 to 500 mg of aminophylline would initially result in about 1000 to 2000 μg/min being delivered to the coronary circulation. This would represent the upper limit of therapeutic blood levels.

To explain the reduction in CBF produced by low dose infusions of aminophylline, we suggested in an earlier study (Paoloni & Wilcken, 1971) that aminophylline may be potentiating α-adrenoceptor mediated effects of catecholamines on coronary vessels leading to coronary vasoconstriction, since aminophylline will potentiate the pressor effects of catecholamines in the peripheral circulation (Bartelstone *et al.*, 1967). The present studies do not support this as there was no change in the low dose aminophylline vasoconstrictor response after α-receptor blockade. But the response might be explained by low dose infusions of aminophylline inhibiting the vasodilator action of adenosine released from the heart (Afonso, 1970a; Paoloni & Wilcken, 1971). Adenosine is a known powerful coronary vasodilator (Wolf & Berne, 1956; Bretschneider, Frank, Bernard, Kochsiek & Scheler, 1959; Afonso, 1970b; Paoloni & Wilcken, 1971, 1972) and may be involved in the physiological regulation of coronary blood flow (Berne, 1963, 1964). Our earlier studies showed that infusions of aminophylline 200 μg/min will produce marked inhibition of the coronary vasodilator action of adenosine in this preparation (Paoloni and Wilcken, 1971). Thus, whilst we have no direct proof that adenosine blockade is responsible for the aminophylline-induced reduction in CBF, the results are at least consistent with the hypothesis that adenosine release from the heart is important for the regulation of coronary blood flow.

The role of β-adrenoceptors in the control of CBF has been studied by Parratt & Grayson (1966) and Parratt (1967) who showed that β-receptor blockade with propranolol decreased CBF in the intact dog heart. They suggested that there was normally a β-receptor mediated coronary vasodilator effect on the coronary circulation and that propranolol had blocked this. In the transplanted heart, we found no change in resting CBF after propranolol in this study and in a previous one (Paoloni & Wilcken, 1971) as also did Boake & Folts (1971) using a similar preparation. Presumably the β-adrenoceptor mediated vasodilator stimulus was removed by denervation at the time of transplantation. In the present studies, the larger dose of propranolol (30 μg/min) produced a small negative inotropic effect due either to the inhibition of β-receptor stimulation by circulating catecholamines (Davis, MacDonald & Mason, 1969) or to a direct depressant effect on the transplanted heart or to both (Whitsitt & Lucchesi, 1967). When the lower dose of propranolol (10 μg/min) was used there was no significant change in myocardial contractility although the β-receptor blocking action was retained. These findings suggest that the negative inotropic effect of the higher dose of propranolol was due to a direct depression of myocardial contractility.

Infusions of aminophylline 1000 μg/min resulted in coronary vasodilatation and an increase in myocardial contractility as assessed by dP/dt and $dP\ dt^{-1}\ IIT^{-1}$. The increase in CBF was not influenced by either the high (30 μg/min) or the low (10 μg/min) dose of propranolol. However, the higher dose of propranolol which depressed myocardial contractility also decreased the aminophylline-induced augmentation of myocardial contractility; but despite this, the CBF response was not affected. The lower dose of propranolol produced β-receptor blockade yet did not change contractility or alter the contractility effects of aminophylline. We concluded therefore that β-receptor blockade did not influence the myocardial actions of aminophylline; and that the results with the different doses of propranolol indicated a dissociation between the positive inotropic action of high doses of aminophylline and the coronary vasodilatation which accompanies it, suggesting that in these doses aminophylline has a small direct coronary vasodilator action.

The findings with β-receptor blockade are in agreement with those of both Cohen *et al.* (1970) in the rat heart and Massingham & Nasmyth (1972) in the frog heart, who found that the positive inotropic effects of aminophylline were not influenced by β-receptor blockade with propranolol. However, Marcus *et al.* (1972) produced β-adrenoceptor blockade in the cat isolated papillary muscle with non-depressant doses of propranolol and reported that this reduced the maximum positive inotropic action of theophylline by 40%. These differences in results cannot be explained at present.

Our results show that when given in a dose which does not alter myocardial contractility, aminophylline reduces CBF and increases coronary vascular resistance in the dog isolated heart and that the response is not mediated through an α-adrenoceptor mechanism. They show further that the increases in CBF, the reductions in coronary vascular resistance and the positive inotropic effects obtained with higher doses of aminophylline are not mediated through β-adrenoceptors. The mechanisms of these effects were not identified in the present studies but presumably the known actions of aminophylline on phosphodiesterase (Sutherland *et al.*, 1968) and calcium transport in cardiac muscle (Nayler, 1967) could have been responsible for the high dose effects; the low dose response could have been produced by inhibition of the vasodilator action of locally produced adenosine.

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THE SELECTIVITY OF β-ADRENOCEPTOR ANTAGONISTS ON CARDIOVASCULAR AND BRONCHODILATOR RESPONSES TO ISOPRENALINE IN THE ANAESTHETIZED DOG

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1 The actions of five β-adrenoceptor antagonists, chosen because of reported differences in their selectivities, were compared using the positive chronotropic, vasodepressor and bronchodilator responses to isoprenaline in anaesthetized dogs.

2 Propranolol was a potent antagonist of the isoprenaline responses in all three systems.

3 Practolol and acebutolol (M & B 17,803) blocked the positive chronotropic responses to isoprenaline to a greater extent than the vasodepressor or bronchodilator responses.

4 Butoxamine and α-methyl dichloroisoprenaline showed the opposite selectivity, blocking the vasodepressor and bronchodilator responses to isoprenaline to a greater extent than positive chronotropic responses. However, both drugs were considerably less potent than the other antagonists studied and their selectivities were less clear-cut than those of practolol or acebutolol.

5 All the antagonists lowered the resting heart rate and to a lesser extent the diastolic blood pressure. The effects of propranolol, practolol and acebutolol on heart rate probably result from cardiac β-adrenoceptor blockade. With butoxamine and α-methyl dichloroisoprenaline, however, the effects on heart rate probably result from a direct cardiodepressant action.

6 The relevance of the results to the problem of the sub-classification of β-adrenoceptors is discussed.

Introduction

β-Adrenoceptor antagonists are now widely used in the therapy of angina pectoris, hypertension and cardiac arrhythmias (Lewis, 1971). The ability to block β-adrenoceptors in cardiac muscle has been considered to be the basis of their therapeutic effect (Fitzgerald, 1969). Some of these drugs, notably propranolol, frequently induce respiratory distress in asthmatic subjects, an effect thought to result from block of β-adrenoceptors in airways smooth muscle (McNeill, 1964; Richardson & Sterling, 1969). This disadvantage is avoided by the use of the 'cardioselective' β-adrenoceptor antagonist, practolol (Dunlop & Shanks, 1968). In clinical trials with asthmatic subjects practolol caused a much lower incidence of respiratory side effects than propranolol (MacDonald & McNeill, 1968; Bernecker & Roetscher, 1970). Pharmacological results with other cardioselective β-adrenoceptor antagonists, for example, acebutolol (M & B 17,803) have been reported more recently (Basil, Jordan, Loveless & Maxwell, 1973).

The cardioselectivity of practolol and similar agents probably results from differences in the nature of the β-adrenoceptors in cardiac and airways smooth muscle. Lands and co-workers suggested that the β-adrenoceptors in cardiac muscle (β_1) differ from those in peripheral vascular and airways smooth muscle (β_2) (Lands, Arnold, McAuliff, Luduena & Brown, 1967; Lands, Luduena & Buzzo, 1967). Subsequent studies with both agonists and antagonists have added support to the concept of β-adrenoceptor sub-types (see Furchgott, 1972).

In the present study, the actions of five β-adrenoceptor antagonists are compared against isoprenaline positive chronotropic, vasodepressor and bronchodilator responses in the anaesthetized dog. The antagonists, chosen because of reported differences in their selectivities, were propranolol, practolol, acebutolol, butoxamine and α-methyl dichloroisoprenaline (α-methyl DCI).

Methods

Preparation of animals

Beagles of either sex weighing 7-13 kg were used. Anaesthesia was induced with thiopentone sodium (25 mg/kg i.v.) and was maintained with barbitone sodium (250 mg/kg i.p.). Animals were respired artificially with room air through a cuffed endotracheal tube using a stroke-volume of 13 ml/kg and a rate of 33/minute. Bilateral vagotomy was performed in all animals. Drugs were administered intravenously through a cannula in a femoral vein. Bilateral vagotomy and the use of a barbiturate anaesthetic reduce or eliminate the influence of autonomic reflexes which can otherwise significantly modify the positive chronotropic and bronchodilator responses to isoprenaline (Boissier, Advenier, Giudicelli & Viars, 1971; Vaughan Williams, Bagwell & Singh, 1973).

Interaction between isoprenaline and β-adrenoceptor antagonists on pulmonary airways resistance

As a first requirement it was necessary to establish a suitable method for measuring the effects of β-adrenoceptor antagonists on isoprenaline 'bronchodilator' responses. In initial experiments the direct effect of isoprenaline on pulmonary airways resistance was measured. Boissier *et al.* (1971) obtained dose-related decreases in pulmonary airways resistance in response to isoprenaline, but in our preliminary experiments the responses obtained were small and dose-response relations were inadequate. This may have been because resting pulmonary resistance was low, as has also been observed in anaesthetized animals by other workers (Lulling, Prignot & Lievens, 1968). Since satisfactory dose-response curves to isoprenaline are obtained by measuring the inhibitory effect of the drug against acetylcholine-induced increases in pulmonary resistance (Daly, Farmer & Levy, 1971), this method was adopted for use in the present experiments.

Pulmonary resistance was measured as described by Daly *et al.* (1971). A dose of acetylcholine, selected to give a submaximal increase of approximately 3-fold in pulmonary resistance, was administered intravenously at 5 min intervals until constant responses were obtained. Isoprenaline was injected intravenously 1 min before the next dose of acetylcholine and further doses of acetylcholine were given repeatedly until constant responses were again obtained. Isoprenaline produced a dose-dependent inhibition of the response to acetylcholine, the maximum effect observed being at the 1 min interval. Responses to isoprenaline, expressed as the percentage inhibition at 1 min of the acetylcholine-induced increase in pulmonary resistance, were plotted against log isoprenaline dose (e.g. Figure 1) in each experiment. Isoprenaline 'bronchodilator' dose-response relations were redetermined, commencing 15 min after each of 3 or 4 intravenous doses of a β-adrenoceptor antagonist, the determination of each set of values taking about 45 min to complete. A cumulative dosing schedule was used; for example, to increase the antagonist dose-level from 0.1 mg/kg to 1 mg/kg a further dose of 0.9 mg/kg was administered.

Whenever possible, quantitative analysis was carried out using the method of Arunlakshana & Schild (1959). The results for each experiment were plotted as log (isoprenaline dose-ratio-1) against log dose antagonist. The regression line was fitted by eye. The slope of the regression and the dose of antagonist (mg/kg) to produce an isoprenaline dose-ratio of 10 were estimated. Providing experimental conditions are satisfactory (Furchgott, 1972), evidence of competitive antagonism is provided by a parallel displacement to the right of the agonist dose-response curve and a slope of unity for the regression line (Arunlakshana & Schild, 1959).

Interaction between isoprenaline and β-adrenoceptor antagonists on heart rate and blood pressure

Arterial blood pressure (1 mmHg ≈ 133 Pa) was measured from a femoral artery with a Bell and Howell physiological pressure transducer connected to a Devices M4 recorder. The pulse pressure was used to trigger a heart rate meter for display. The experimental design was similar to that described above. Isoprenaline dose-response relations for increase in heart rate (beats/min) and decrease in diastolic blood pressure (mmHg) were determined by the sequential intravenous injection of increasing doses of the agonist before and from 15 min after intravenous administration of the antagonist. Each set of determinations took about 45 min to complete. Absolute changes in heart rate and blood pressure were used for analysis as recommended by Vaughan Williams *et al.* (1973). The magnitude of each response was usually estimated by comparing the peak level with the immediately preceding resting level. However, a difficulty arose in experiments with the cardioselective antagonists practolol and acebutolol. In these, the large doses of isoprenaline required to re-establish positive chronotropic responses caused falls in diastolic blood pressure that persisted between isoprenaline doses. In these instances the resting level immediately before the first dose of antagonist was used for comparison. Quantitative analysis of these results was performed as in the preceding section.

The effects of the β-adrenoceptor antagonists on resting heart rate and blood pressure were assessed at 15 min after injection i.e. immediately before determining sensitivity to isoprenaline.

Drugs

The following drugs were used: acebutolol hydrochloride (May & Baker Ltd), acetylcholine chloride (BDH Chemicals Ltd), barbitone sodium (BDH Chemicals Ltd), butoxamine (Wellcome Research Laboratories), α-methyl dichloroisoprenaline (A.B. Hassle), (±)-isoprenaline sulphate (Burroughs Wellcome and Co.), (±)-practolol (Imperial Chemical Industries), (±)-propranolol hydrochloride (Imperial Chemical Industries), thiopentone sodium (Abbott Laboratories Ltd).

Unless stated otherwise, dosages are expressed in terms of free base.

Results

Effects on sensitivity to isoprenaline

In control experiments, determinations of positive chronotropic, vasodepressor and bronchodilator dose-response relations to isoprenaline at hourly intervals for 6 h showed that the sensitivity of individual dogs to isoprenaline varied by less than 3-fold over this period. Representative experiments using propranolol are illustrated in Figure 1 and results using various antagonists are summarized in Table 1.

Propranolol. Propranolol (0.01, 0.1 and 1 mg/kg) produced parallel displacements to the right of the positive chronotropic, vasodepressor and bronchodilator dose-response curves for isoprenaline. Small differences in the potency of propranolol were noted in the three tissues. Thus, propranolol was about four times more potent in blocking vasodepressor than positive chronotropic responses to isoprenaline ($P < 0.02$) and tended to be more potent in blocking bronchodilator than cardiac responses ($P > 0.05$).

Practolol. Practolol (0.3, 3 and 30 mg/kg) produced parallel displacements to the right of the isoprenaline positive chronotropic dose-response curve, but in the same experiments had little or no effect on vasodepressor responses to isoprenaline in doses of 0.3 or 3 mg/kg. At 30 mg/kg practolol produced a 3-fold displacement to the right of the isoprenaline vasodepressor dose-response curve in three out of four experiments, but was ineffective in the fourth experiment.

Practolol also had low potency against isoprenaline bronchodilator responses. Thus, there was little or no effect in doses up to and including 10 mg/kg. Higher doses of practolol (30 and 100 mg/kg) displaced the isoprenaline bronchodilator dose-response curve to the right. Practolol was approximately 80 times more potent in blocking positive chronotropic responses to isoprenaline than bronchodilator responses.

Acebutolol. Acebutolol (0.03, 0.3 and 3 mg/kg) produced parallel displacements to the right of the positive chronotropic dose-response curve to isoprenaline. It had no effect on vasodepressor responses to isoprenaline at 0.03 and 0.3 mg/kg but at 3 mg/kg displaced the curve to the right by 2 to 4-fold in three out of four experiments; the fourth experiment showed no antagonism. Bronchodilator responses to isoprenaline were not impaired by acebutolol, 0.3 mg/kg but 3 and 30 mg/kg displaced the dose-response curve to the right. Acebutolol was approximately 60 times more potent in blocking positive chronotropic responses to isoprenaline than bronchodilator responses.

Butoxamine. Butoxamine, in contrast to the above antagonists, had no effect on isoprenaline positive chronotropic responses in doses up to and including 10 mg/kg and was lethal at 30 mg/kg. It had no effect on isoprenaline vasodepressor responses in doses up to and including 1 mg/kg but at 3 mg/kg reduced the sensitivity to isoprenaline by 7-fold in one experiment, while producing no effect in two other experiments. At 10 mg/kg the isoprenaline dose-response curve was displaced to the right in all three dogs by factors of 10-60-fold. The low doses of isoprenaline (0.1-1 μg/kg) that previously had produced depressor responses now produced small pressor responses (10 mmHg). Butoxamine had no effect on isoprenaline bronchodilator responses at 0.1 and 1 mg/kg but at 10 mg/kg reduced the sensitivity by 7-10-fold in each of three experiments.

α-Methyl DCI. Only a small amount of this drug was available for study. It had no effect on positive chronotropic responses to isoprenaline at 0.3 and 3 mg/kg but at 30 mg/kg reduced the sensitivity by 5-fold in one experiment and by

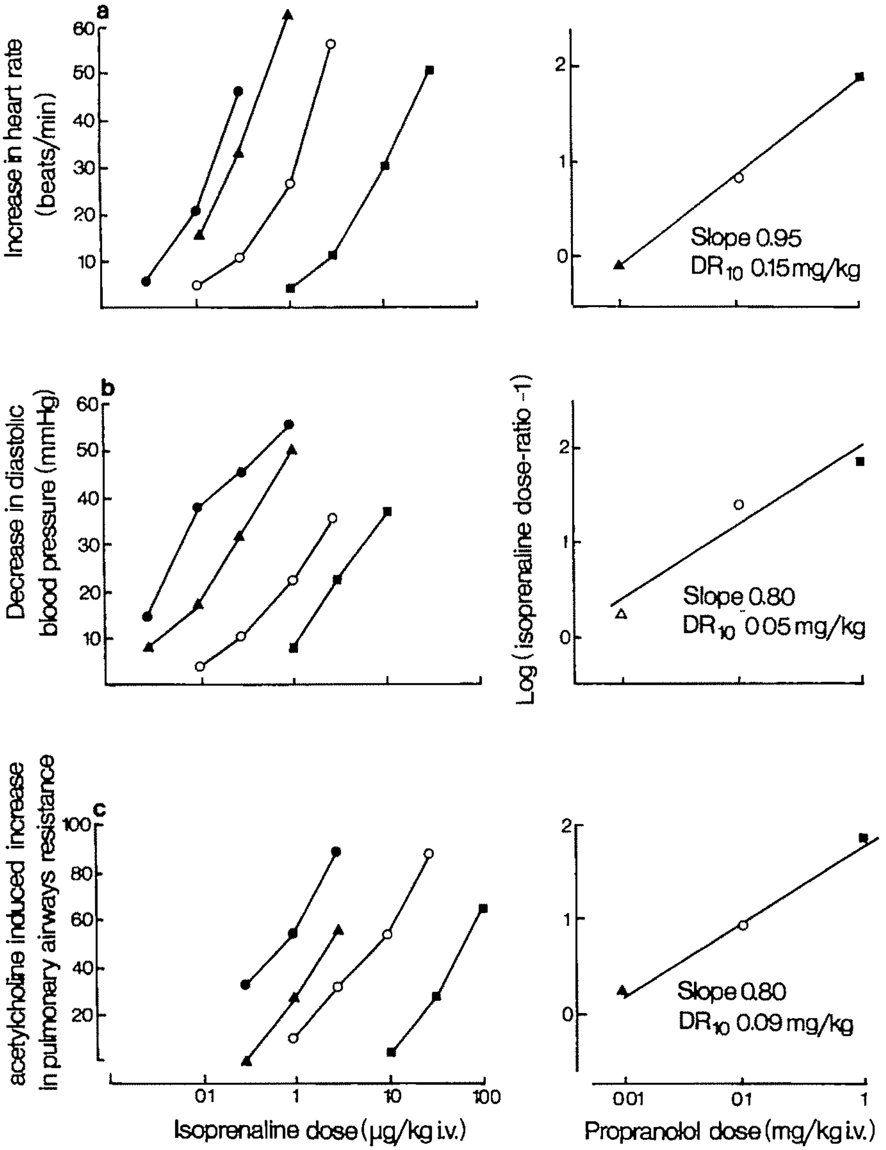

Figure 1 Effects of propranolol on responses of (a) heart rate, (b) blood pressure and (c) pulmonary airways resistance to isoprenaline in the anaesthetized dog. (●) Pre-dose; (▲) 0.01 mg/kg; (○) 0.1 mg/kg; (■) 1 mg/kg propranolol i.v. (a) and (b) determined in the same animal. DR$_{10}$ — as in Table 1.

10-fold in another. There was no effect on the vasodepressor responses at 0.3 mg/kg, but reduced sensitivity to isoprenaline at 3 and 30 mg/kg. Reversal of the isoprenaline vasodepressor responses was not observed. Sensitivity to the bronchodilator responses was unaffected by 1 mg/kg and reduced by 3 and 10 mg/kg.

Effects on resting heart rate, diastolic blood pressure and pulmonary resistance

All the antagonists tested lowered resting heart rate and diastolic blood pressure (Table 2), effects on heart rate being the more pronounced. Propranolol, practolol and acebutolol each

Table 1 Effects of β-adrenoceptor antagonists on positive chronotropic, vasodepressor and bronchodilator responses to isoprenaline in the anaesthetized dog

	Positive chronotropic response			*Vasodepressor response*			*Bronchodilator response*†		
Antagonist	*DR_{10}*	*Slope*	n	*DR_{10}*	*Slope*	n	*DR_{10}*	*Slope*	n
Propranolol	0.13 (0.09-0.17)	1.18 (0.85-1.51)	4	0.03 (0.01-0.04)	1.03 (0.73-1.33)	4	0.06 (0.03-0.15)	0.83 (0.70-0.96)	4
Practolol	0.54 (0.35-0.82)	0.94 (0.74-1.14)	4	>30 (3-fold displacement at 30 mg/kg in 3 of 4 expts.)	—	4	44 (range 28-56)	1.25 (range 0.83-1.58)	3
Acebutolol	0.30 (0.24-0.39)	0.91 (0.81-1.01)	4	>3 (2-4-fold displacement at 3 mg/kg in 3 of 4 expts.)	—	4	18.6 (range 12-35.5)	1.14 (range 1.05-1.22)	3
Butoxamine	>10 (No block at 10 mg/kg; lethal at 30 mg/kg)	—	3	Blocks at 10 mg/kg (Responses to low doses of isoprenaline reversed; responses to higher doses reduced)	—	3	c. 10 (No block at 1 mg/kg; 7-10-fold displacement at 10 mg/kg in 3 expts.)	—	3
α-Methyl DCI	c. 30 (No block at 3 mg/kg; 5 and 10-fold displacement at 30 mg/kg in 2 expts.)	—	2	13.0	0.60	1	9 (range 3-13)	1.00 (range 0.75-1.40)	3
				13.5	0.65	1			

DR_{10} — dose of antagonist (mg/kg i.v.) to produce an isoprenaline dose-ratio of 10

Slope — of regression obtained from plot of log (isoprenaline dose-ratio—1) on log (antagonist dose, mg/kg i.v.)

n — number of experiments

Figures in parentheses are 95% confidence limits unless otherwise indicated

† — inhibition by isoprenaline of the acetylcholine-induced increase in pulmonary airways resistance

Table 2 Effects of β-adrenoceptor antagonists on heart rate and diastolic blood pressure in the anaesthetized dog

Antagonist	*Dose (mg/kg i.v.)*	*Decrease in heart rate (beats/min)*	*Change in diastolic blood pressure (mmHg)*
Propranolol		[Pre-dose, 133(110-150)]	[Pre-dose, 90(70-120)]
(*n* = 4)	0.01	14(5-20)	+1 (+10 to −10)
	0.1	31(10-45)	−2.5(+5 to −20)
	1	46(15-70)	−7.5(−5 to −20)
Practolol		[Pre-dose, 143(135-155)]	[Pre-dose, 101(87-115)]
(*n* = 4)	0.3	26(25-30)	−8 (0 to −15)
	3	42(40-45)	−7 (0 to −22)
	30	57(55-59)	−11(−5 to −20)
Acebutolol		[Pre-dose, 147(116-170)]	[Pre-dose, 98(55-115)]
(*n* = 4)	0.03	20(3-35)	−2.5(0 to −10)
	0.3	38(15-60)	−15 (−5 to −30)
	3	54(31-75)	−26 (−15 to −40)
Butoxamine		[Pre-dose, 172(150-185)]	[Pre-dose, 122(110-135)]
(*n* = 3)	1	8(5-10)	0
	3	26(20-35)	−5 (0 to −10)
	10	68(50-75)	−30(−15 to −45)
	30	Lethal	
α-Methyl DCI		[Pre-dose, 150-155]	[Pre-dose, 77-115]
(*n* = 2)	0.3	5-11	−2 to −5
	3	23-25	+1 to −15
	30	55-58	−5 to −15

Pre-dose—level of heart rate or diastolic blood pressure immediately before first dose of β-adrenoceptor antagonist
n-number of experiments
Values in brackets are ranges
Data in this Table and Table 1 obtained from the same experiments.

lowered heart rate in dose ranges found to block isoprenaline positive chronotropic responses. In contrast, butoxamine and α-methyl DCI lowered heart rate in doses that had little or no effect on positive chronotropic responses to isoprenaline. None of the antagonists had any significant effect on resting pulmonary airways resistance.

Discussion

The present work involved two series of experiments—one measuring antagonist potency against isoprenaline bronchodilator responses and the other against isoprenaline positive chronotropic and vasodepressor responses. This was necessary because the doses of acetylcholine used to produce bronchoconstriction also caused pronounced cardiovascular effects. Comparisons of drug potency from one animal to another are complicated by individual variations in sensitivity but variation was relatively small in the present experiments (Table 1). The main advantage of the methods used is that accurate determinations of antagonist potency could be obtained in each of the three systems examined.

The results with propranolol confirm that the drug is a potent antagonist of positive chronotropic, vasodepressor and bronchodilator responses to isoprenaline. The observation that vasodepressor responses were blocked to a slightly greater degree than positive chronotropic responses is in agreement with previous findings (Shanks, 1966; Boissier *et al.*, 1971; Vaughan Williams *et al.*, 1973).

We found practolol to be about four times less potent than propranolol in antagonizing isoprenaline positive chronotropic responses, in accord with previous estimates in animals and in man (Barrett, Crowther, Dunlop, Shanks & Smith, 1968; Brick, Hutchison, McDevitt, Roddie & Shanks, 1968; Wale, Pun & Rand, 1969; Boissier *et al.*, 1971; Burden & Parkes, 1971; Basil *et al.*, 1973). In contrast, practolol had low antagonist potency against isoprenaline vasodepressor and bronchodilator responses. Although there is agreement in the literature that practolol has a relatively greater potency on cardiac than on vascular responses (Barrett, *et al*, 1968; Dunlop & Shanks, 1968; Wale *et al.*, 1969; Boissier *et al.*, 1971; Vaughan Williams *et al.*, 1973), there is conflicting evidence regarding its relative effects on cardiac and pulmonary responses. Thus, whereas there are reports that practolol has no specificity in this respect in guinea-pigs (Burden & Parkes, 1971) and dogs (Boissier *et al.*, 1971) it has also been claimed that practolol has a relatively greater effect on cardiac responses in both guinea-pigs (Baird & Linnell, 1972) and cats (Parkes, 1973). The reasons for these differences are not known; the degree of selectivity could vary somewhat depending on the species and the method of assessment used (Basil *et al.*, 1973). However, our results and the other reports showing a significant cardiac to pulmonary selectivity with practolol would seem to be in accord with findings in asthmatic subjects (see Introduction). The observed cardio-selectivity of acebutolol was comparable with that of practolol as in other studies using anaesthetized dogs (Briant, Dollery, Fenyvesi & George, 1971; Basil *et al.*, 1973) but Baird & Linnell (1972) found this drug to be much less cardioselective in guinea-pigs and there is doubt about its selectivity in man (see Basil *et al.*, 1973; Briant, Dollery, Fenyvesi & George, 1973).

Butoxamine was found to be a weak β-adrenoceptor antagonist, but with some degree of selectivity. Thus, it prevented isoprenaline vasodepressor responses at 10 mg/kg without modifying positive chronotropic responses. After butoxamine, small doses of isoprenaline exerted a pressor effect attributed to its cardiac stimulant action not being masked by vasodilatation. These results confirm previous reports that butoxamine blocks vasodepressor, but not cardiac responses to isoprenaline (Levy, 1966; Wilkenfeld & Levy, 1968; Parratt & Wadsworth, 1970). The present experiments show for the first time that butoxamine blocks isoprenaline bronchodilator responses in the dose-range required to block the vasodepressor responses. The examination of butoxamine was limited to a relatively narrow dose-range because the drug was lethal at 30 mg/kg. This may derive from a direct cardiodepressant action, suggested by the finding that butoxamine caused large decreases in heart rate and has previously been shown to markedly decrease cardiac contractility (Levy, 1966; Wilkenfeld & Levy, 1968; Wastila, Su, Friedman & Mayer, 1972).

Like butoxamine, α-methyl DCI was more effective in blocking isoprenaline vasodepressor and bronchodilator responses than positive chronotropic responses. The vascular to cardiac selectivity of α-methyl DCI has been reported previously (Moran, 1966). α-Methyl DCI also resembled butoxamine in that doses that decreased resting heart rate failed to block positive chronotropic responses to isoprenaline, indicating a direct cardiodepressant action.

Different profiles of selectivity are apparent in the β-adrenoceptor antagonists examined. Several workers have related similar data, also obtained in the anaesthetized dog, to differences in the β-adrenoceptors mediating the observed responses. Levy & Wilkenfeld (1969) concluded that cardiac and intestinal smooth muscle β-adrenoceptors belong in one category (β_1) and peripheral vascular β-adrenoceptors in another (β_2), in accord with Lands and co-workers dual β-adrenoceptor classification (see Introduction). Other findings (Boissier *et al.*, 1971; Levy, 1973a, b, c; Wasserman & Levy, 1974), would seem to indicate that β-adrenoceptors in peripheral vascular and airways smooth muscle are different, which is not in accord with the Lands classification.

Experiments in intact animals have undoubtedly been helpful in receptor classification, but there are certain drawbacks to their use, especially when attempting to differentiate between receptors within a single class. Particularly rigorous control of experimental conditions is then necessary and this cannot be achieved in the intact animal (Furchgott, 1972). For example, it is not possible to control the distribution and disposition patterns of the administered drugs. Conclusions from the above publications are further complicated because some of the antagonists used had only low potency in the tissues concerned (Levy, 1973c; Wasserman & Levy, 1974) or because some results were inconsistent with competitive antagonism (Boissier *et al*, 1971). With these potential drawbacks in mind, it is appropriate to consider the relevance of the present results to the sub-classification of β-adrenoceptors.

Propranolol was shown to be a potent but non-selective antagonist. Practolol and acebutolol showed selectivity for cardiac compared with peripheral vascular and airways smooth muscle. Butoxamine and α-methyl DCI showed the opposite selectivity, although this was less clear-cut. These findings provide strong support for the idea that β-adrenoceptors in cardiac muscle differ from those in peripheral vascular and airways smooth muscle. The question of the relationship between β-adrenoceptors in the latter two tissues is more difficult to answer because of the low potency of butoxamine and α-methyl DCI. A potent antagonist of this type would be a useful aid in the sub-classification of β-adrenoceptors. However, the results do show that although there were clear differences in the potencies of the five antagonists examined, the potency of each was similar in peripheral vascular and airways smooth muscle. This tends to favour the idea that the β-adrenoceptors in these tissues are of the same type. A similar conclusion was drawn from a corresponding study with the selective β-adrenoceptor agonist salbutamol (Daly *et al.*, 1971). Thus, these findings, unlike some cited above, are consistent with the Lands dual β-adrenoceptor classification.

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THE INHIBITORY ACTION OF OESTRADIOL-17-β AND PROGESTERONE ON VENOUS SMOOTH MUSCLE

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1 The *in vitro* action of oestradiol-17-β (0.1, 1.0 and 10.0 μg/ml) and progesterone (0.1, 1.0 and 10.0 μg/ml) on the spontaneous activity of the portal veins in female rats at different stages of gestation was studied.

2 Progesterone caused the spontaneous mechanical activity in the rat portal vein to decrease in amplitude and increase in frequency. This action was dose-dependent and the sensitivity of the tissue decreased throughout pregnancy.

3 Oestradiol-17-β had a biphasic effect on spontaneous mechanical activity in the rat portal vein. At 0.1 μg/ml the vessel was stimulated while a similar effect to progesterone occurred with higher concentrations. The tissue was more sensitive to oestradiol at 7 days of gestation than at either the 10-14 or 17-21 day periods of gestation.

4 These effects of oestradiol and progesterone were still seen after blockade of the β-adrenoceptors.

5 Hydrocortisone (10 μg/ml) had no inhibitory effect on the spontaneous mechanical activity of the vein.

6 The veins from the 17-21 days pregnant animals showed a smaller amplitude of contraction than comparable non-pregnant females.

Introduction

Virchow (1856) made the observation that there were three major factors to be considered in the pathogenesis of venous thrombosis. These were, damage to the endothelium, an increase in blood coagulability and stasis of blood in the veins. Varicosity is a condition which could cause stasis of the blood in the veins thus contributing one factor towards Virchow's 'triad'. Wessler (1962) showed that when blood was mechanically stopped in superficial veins there was some thrombus formation after 30 minutes. Thus stasis is a powerful factor contributing towards thrombosis.

The occurrence of varicose veins and thrombophlebitis during pregnancy (Aaro & Juergens, 1971) and during oral contraceptive therapy (Grant, 1969) suggests that the steroids oestradiol-17-β and progesterone (which are elaborated in large amounts during pregnancy) may be responsible. The present experiments were to test the theory that these hormones directly cause inhibition of the venous smooth muscle and consequent dilatation of veins. The mechanism of inhibition was also investigated.

The tissue chosen for this investigation was an *in vitro* preparation of the rat portal vein. Thrombosis of mesenteric veins has been reported in conjunction with the use of orally administered steroids (Bergain, 1969; Hurwitz, Martin, Grossman & Waddel, 1970). Thus, any findings can be used to assess the role of the steroids in the aetiology of this type of complaint.

Methods

White Wistar rats weighing between 200-350 g were killed with a sharp blow on the back of the skull. The abdomen was then opened and the portal vein was located and exposed. Ligatures were placed around the vein at the point of its entry into the liver and at its junction with the superior mesenteric vein. This portion of the vein along with the ligatures was 2.5-3.0 cm long and was removed to a beaker containing modified Krebs (1950) solution of the following composition (mM): NaCl 118, KCl 4.69, $NaHCO_3$ 25.0, NaH_2PO_4 1.33, glucose 5.56, $CaCl_2$ 2.52 and

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$MgCl_2$ 1.05. The vessel was gently cleared of surrounding adipose tissue and was mounted vertically in a 10 ml organ bath. The bath was perfused at 15-20 ml/min with fresh Krebs solution which was prewarmed to 37°C and pre-oxygenated with 95% O_2 and 5% CO_2. The bath was surrounded by a water jacket through which water at 37°C was pumped.

The vein was stretched and relaxed two or three times and finally stretched until its length was approximately the *in vivo* length and the tension was approximately 0.25 g (2.5 mN). A period of equilibration was then allowed while the muscle relaxed and began to contract spontaneously. These contractions were recorded with an isometric force transducer and a pen recorder (Devices). Usually 30 min was long enough to allow a regular pattern of activity to be established. Four separate series of experiments were carried out with this preparation. Their protocols are described below.

(A) *Oestradiol and progesterone*

Thirty-four experiments were carried out in this group. The portal vein was removed and set up as previously described. After the initial period of equilibration the spontaneous mechanical activity was recorded during the following four consecutive periods: (i) 30 min—Normal Krebs perfusate; (ii) 2 h—0.1 μg/ml steroid perfusate; (iii) 2 h—1.0 μg/ml steroid perfusate, (iv) 2 h—10 μg/ml steroid perfusate. This protocol was used because it was difficult to reverse the effect of the steroid by washing with normal Krebs solution. In order to test three concentrations of steroid on each preparation and still keep the experimental time short this form of cumulative dose response trial was used. Ethanol (1 ml/l) was used throughout to make the steroids water soluble.

The wet weight of each vein was determined at the end of each experiment and all measurements of force produced by each preparation were expressed as milliNewtons/mg wet weight of tissue. (10 mN ≙ 1 g).

The frequency of contraction and the average amplitude of contraction/mg of tissue were measured during the last 10 min of each experimental period. The total contractile activity was estimated by integration of the trace of contractions against time. This was useful as an overall indicator of total activity in that an increase in the frequency of contraction, the amplitude of contraction or the duration of contraction produced an increase in the integral. The calculation was done automatically with a Resistance-Capacitor operational amplifier. The input to the integrator was balanced so that the resting tension in the preparation did not affect the integral. The capacitor was automatically discharged every minute.

Twelve young anoestrous female rats were used as a baseline group. Seven of these were used in a progesterone experiment and five in an oestradiol experiment. The remaining 22 animals were used for experiment at stages of pregnancy. Vaginal washings were examined daily and the day when sperm were found was designated as day 1 of pregnancy. Six animals were used at 7 days of gestation (3 for progesterone and 3 for oestradiol). Eight animals were used at 10-14 days of gestation (5 for progesterone and 3 for oestradiol). Eight animals were used at 17-21 days of gestation (5 for progesterone and 3 for oestradiol).

The effects of the steroids was assessed by noting the changes produced in the amplitude of contraction, the average frequency of contraction and the total contractile activity. The significance of any of these changes was tested by a paired *t* test.

The effects of pregnancy were assessed by noting the differences between these measurements during the initial 30 min perfusion period in Krebs solution over the four groups of animals. The significance of any difference was tested with a *t* test.

(B) *The controls*

To establish that any change in spontaneous activity of a preparation was due to the steroid perfusate and not due to temporal changes in the muscle or to ethanol, a total of eight control experiments were carried out with rats from each of the pregnancy groups and the portal veins were removed and set up as previously described. The spontaneous mechanical activity was recorded while the bath was perfused with fresh Krebs solution plus 1 ml/l of steroid-free ethanol. This was often done concurrently with a steroid experiment and the measurements and calculations were made in the same way.

(C) *The effects of β-adrenoceptor blockade*

Six experiments were carried out in this section. Oestradiol was used in three of these and progesterone in the others. The effects of isoprenaline (Boots) were first tested by injecting known amounts (1 μg and 10 μg) of the drug into the bath along with the perfusate. Washing out was automatic because the bath was continuously perfused with fresh Krebs solution. When the effects of β-adrenoceptor stimulation had been observed, the perfusate was changed to one

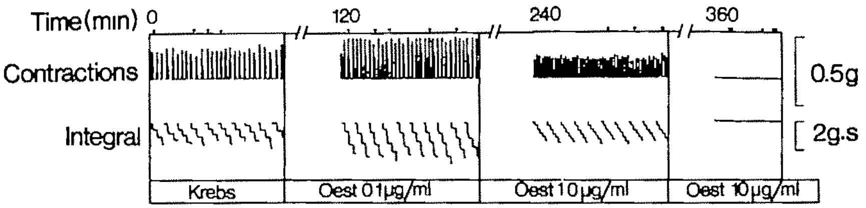

Figure 1 The spontaneous mechanical contractions and the minute integral of the portal vein from a young non-pregnant female rat. The block of records on the left shows the pattern of activity after equilibration in Krebs solution. The other three blocks show the effects of oestradiol (Oest) in concentrations of 0.1, 1.0 and 10.0 µg/ml when added cumulatively for periods of 2 h each.

containing propranolol 1.0 µg/ml (I.C.I.) and the tissue left for 30 minutes. β-Adrenoceptor blockade was confirmed by challenging the tissue with the same amounts of isoprenaline. Then either progesterone (10 µg/ml) or oestradiol (10 µg/ml) was added to the perfusate and the effect on the spontaneous mechanical activity was observed.

(D) *The effects of hydrocortisone*

Three experiments were carried out in this group. After a period of equilibrium and recording of normal activity, hydrocortisone (10 µg/ml) was added to the organ bath perfusate and the spontaneous activity was observed for two hours.

Results

The effects of oestradiol and progesterone on the spontaneous mechanical activity of portal veins from non-pregnant rats

Oestradiol-17-β Figure 1 shows the effect of cumulative addition of oestradiol in concentrations of 0.1, 1.0 and 10.0 µg/ml to the bath containing a rat portal vein. This portal vein was taken from a young anoestrous female rat. On the left of the figure is a block of records taken while the tissue was exposed to normal Krebs solution. A time base with one pulse per min is shown at the top. The trace beneath the time base shows the spontaneous contractions of the tissue as an upwards deflection of the pen. The integral of this contraction/time curve was determined electronically and is shown on the bottom trace. The integral is an estimate of the area under the curve and the total contractile activity of the tissue. This value is shown as a downwards deflection of the bottom trace and is reset to zero every minute. The three blocks of recording to the right of the first are similar records of the portal vein contractions and the integral of the contractions taken at the end of three consecutive 2 h periods when perfusates containing oestradiol at 0.1, 1.0 and 10.0 µg/ml were used. Calibrations for the pen deflections are shown on the extreme right and apply to all traces on the figure.

Figure 1 shows that during the initial period of Krebs perfusion there was a regular pattern of activity. The average amplitude of contraction was approximately 0.2 g and these occurred at a frequency of 2-3/minute. The integral on the lower trace shows that there was very little variation in the total contractile activity, the average in each minute being approximately 1.5 g.second. When the tissue was exposed to a perfusate containing 0.1 µg/ml of oestradiol for 2 h, the pattern of activity changed to that shown in the second block of traces. There were increments in the amplitude of contraction and the frequency of contraction. The integral shows that there was an increase in the total contractile activity to approximately 2.5 g.second. The third block of records in Figure 1 shows the activity after a 2 h period of exposure to oestradiol at 1.0 µg/ml. At this concentration a reduction in the amplitude of contraction to below the initial value in Krebs solution occurred and although the frequency of contraction simultaneously increased, the resultant total contractile activity in each minute was much reduced to approximately 1.0 g.second. A further increase in the concentration of the oestradiol to 10.0 µg/ml caused further large reductions in the activity of the muscle. The fourth block of records shows that after exposure to 10.0 µg/ml oestradiol for two hours the activity is of low amplitude but high frequency. Thus oestradiol seems to have a biphasic effect. At low concentrations the muscle of the rat portal vein is stimulated to stronger contraction. After 2 h of oestradiol at 1.0 µg/ml there was a significant decrease in the average amplitude of contraction ($P < 0.05$), an increase in the number of contractions ($P < 0.05$) and a decrease in total

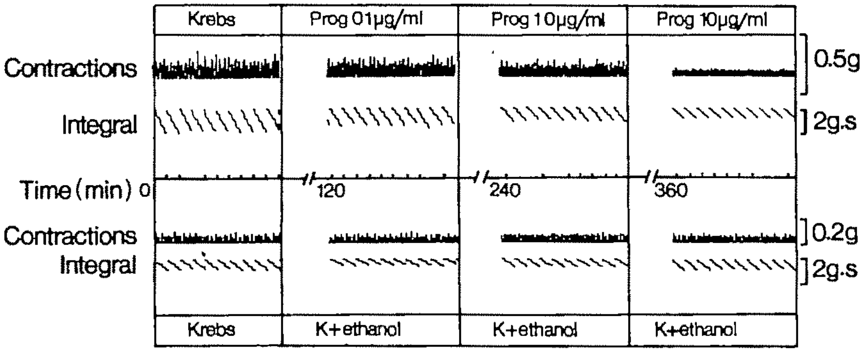

Figure 2 The spontaneous mechanical contractions and the minute integral of two portal veins from two young non-pregnant female rats. Above the time base are shown the records when progesterone (Prog) at concentrations of 0.1, 1.0 and 10.0 μg/ml were added cumulatively for periods of 2 h each. Below the time base are shown the records when no steroid was added to the bath. This was recorded simultaneously with the progesterone experiment above and a perfusate of Krebs plus ethanol (1 ml/litre) was used throughout.

contractile activity ($P < 0.01$). These effects were further accentuated by the use of oestradiol at 10 μg/ml for a further 2 h ($P < 0.01$). These P values are based on 5 experiments with anoestrous rats.

Progesterone Figure 2 shows two experiments similar to the previous oestradiol experiment. Above the time base (one pulse/min) are shown the records when progesterone at concentrations of 0.1, 1.0 and 10.0 μg/ml were cumulatively perfused through the organ bath for 2 h each. The records on the extreme left show the contraction pattern and the minute integral when the tissue was exposed only to Krebs solution. The average amplitude of contraction was approximately 0.5 g. and these occurred 3-4 times per minute. The integrals show that the total contractile activity produced was between 1.5-2.0 g.second. When progesterone at a concentration of 0.1 μg/ml was introduced to the tissue bath the contractile activity did not noticeably change. The effects of progesterone 1.0 μg/ml are shown in the third group of records. There was a noticeable decrease in the amplitude of the contractions and an increase in the frequency of contraction. The integral showed that overall there was a decrease in the total contractile activity. Further elevation of the progesterone concentration to 10.0 μg/ml caused the same effects but to a greater degree.

Seven experiments were carried out when cumulative dose responses of portal veins from anoestrous female rats to progesterone were investigated. After 2 h of exposure to 0.1 μg/ml there was no significant change in any of the variables. An increase in the dose to 1.0 μg/ml caused a significant reduction in the average amplitude of contraction ($P < 0.05$), a significant increase in the number of contractions ($P < 0.05$) and a significant decrease in total contractile activity ($P < 0.05$). These effects were further accentuated by the use of progesterone at 10 μg/ml for a further 2 h ($P < 0.01$).

Controls No attempt was made in either the oestradiol or progesterone experiments of Figures 1 and 2 to return the tissue to its orginal level of activity by washing with a steroid-free perfusate. Also no account was taken of the possible independent effects of ethanol (1 ml/l) which was used to make the steroids water soluble. Therefore a series of control experiments was carried out to show that the tissue was capable of sustaining regular activity without any change in amplitude or frequency when Krebs solution plus steroid-free alcohol was perfused for seven hours. One such experiment is shown below the time base in Figure 2. It was simultaneously carried out with the progesterone experiment above the time base and shows very little change in the pattern of activity over the same period as the concurrent progesterone experiment. Thus any change observed in the spontaneous activity of the portal veins was not due to temporal deterioration of the tissue or to ethanol.

Eight of these control experiments were carried out. No significant changes were observed after 4 h of perfusion with Krebs plus steroid-free alcohol ($P > 0.05$). After 6 h the only variable observed to change was the average amplitude of contraction. This showed a decrease from 0.59 ± 0.05 mN/mg to 0.42 ± 0.00 mN/mg and this was found to be significantly different ($P < 0.05$). These control experiments show that there is some temporal deterioration of the tissue. However this deterioration was small and occurred simultaneously with the enormous decrease in activity caused by 10 μg/ml of steroid. There was no temporal deterioration before that. Thus the changes which occurred during consecutive exposure of a rat portal vein to increasing concentrations of either oestradiol or progesterone were not due to temporal deterioration of the tissue but to the changes in the perfusate.

Pregnant rats—sensitivity to oestradiol and progesterone

Oestradiol and progesterone were applied in similar cumulative concentrations to the portal veins of rats at various stages of gestation. The same variables were measured and the results at the end of each 2 h period of steroid perfusion were expressed as a percentage of the value in the initial 30 min control period when Krebs was used. Figures 3, 4 and 5 show these percentage results averaged over a number of experiments at different stages of gestation.

Total contractile activity Figure 3 shows the total contractile activity after progesterone or oestradiol. The dose-response curves for progesterone are shown on the left and those for oestradiol on the right. The closed circles represent the results from the non-pregnant animals. The open circles represent the results from the 7 day pregnant animals. Open squares represent the results from the 10-14 day pregnant animals and the closed squares represent the 17-21 day pregnant animals.

The progesterone curves (Figure 3a) show that the steroid produced a decrease in the total contractile activity which is dose-dependent at all times during gestation. The curves for the non-pregnant, 7 day and 10-14 day pregnant animals are overlapping, indicating no difference in sensitivity of the venous muscle to the steroid. The curve found for the animals at the end of pregnancy was displaced to the right of this group. This indicates that there was a decrease in the sensitivity of the tissue at this time.

The oestradiol curves (Figure 3b) show that oestradiol has a biphasic effect. At a concentration of 0.1 μg/ml all groups of animals demonstrate an increase in total contractile activity. This was reversed after 1.0 μg/ml and a marked decrease to almost zero was produced after 10.0 μg/ml. Peak sensitivity to the oestradiol was exhibited by the animals which were 7 days pregnant (open circles).

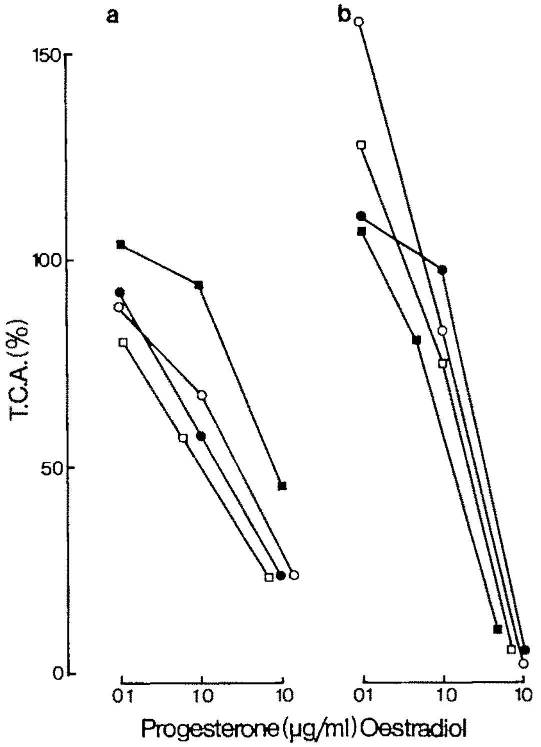

Figure 3 Total contractile activity (T.C.A.)—the % value was obtained by calculating 100 x T.C.A. (steroid)/T.C.A. (presteroid) Dose-response curves are shown for the portal veins from non-pregnant rats (closed circles), 7 days pregnant rats (open circles), 10-14 days pregnant rats (open squares) and 17-21 days pregnant rats (closed squares).

Average amplitude of contraction Figure 4 shows the changes caused by progesterone and oestradiol in average amplitude of contraction at various stages of pregnancy. The dose-response curves are again expressed as a percentage of control values in Krebs solution.

The progesterone curves (Figure 4a) show a dose-dependent decrease in the average amplitude of contraction. The sensitivity seems to be different at different stages of gestation. The non-pregnant and 7 days pregnant animals have similar sensitivities. The 10-14 day pregnant animals show an increased sensitivity while the 17-21 day pregnant animals show a decreased sensitivity.

The oestradiol curves (Figure 4b) show a biphasic response similar to that for total contractile activity. The time of greatest sensitivity was again at 7 days of gestation.

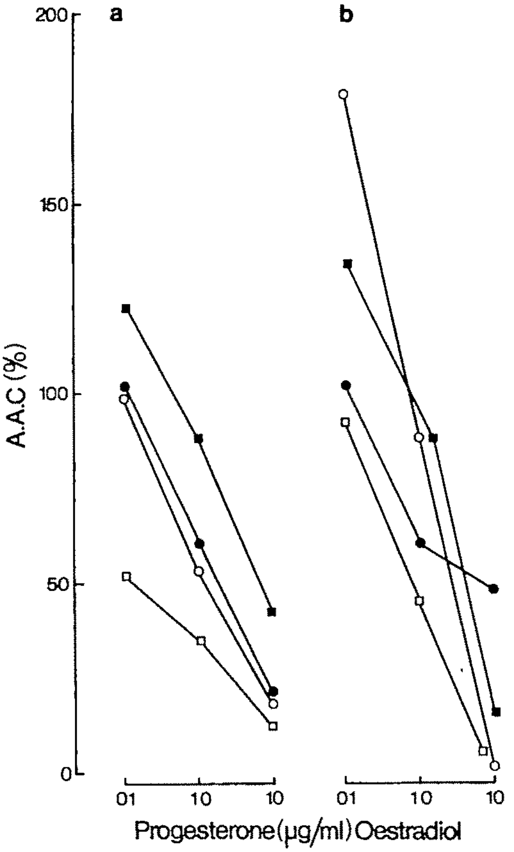

Figure 4 Average amplitude of contraction (A.A.C.)—the % value was obtained by calculating 100 x A.A.C. (steroid)/A.A.C. (presteroid). Dose-response curves are shown for the portal veins from non-pregnant rats (closed circles), 7 days pregnant rats (open circles), 10-14 days pregnant rats (open squares) and 17-21 days pregnant rats (closed squares).

Number of contractions/10 minutes Figure 5 shows similar dose-response curves for the effect of progesterone and oestradiol on the number of contractions per 10 minutes.

The progesterone curves (Figure 5a) show that there is an increase in the frequency of contraction after progesterone and that this effect was dose-dependent. The oestradiol curves (Figure 5b) show a similar pattern. With one exception, an increase in the rate occurred after oestradiol and this increase was dose-dependent. The exception was seen after oestradiol 10 µg/ml in the group of animals which were 7 days pregnant. A decrease in the rate of contraction to zero occurred at this point.

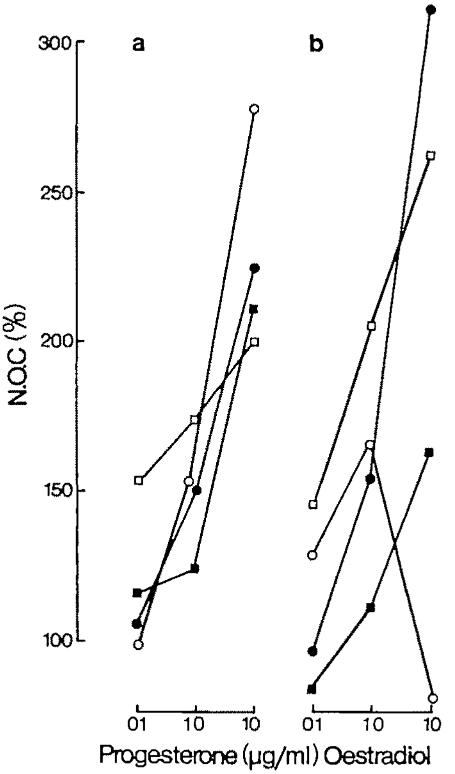

Figure 5 Number of contractions (N.O.C.)/10 minutes—the % value was obtained by calculating 100 x N.O.C. (steroid)/N.O.C. (presteroid). Dose-response curves are shown for the portal veins from non-pregnant rats (closed circles), 7 days pregnant rats (open circles), 10-14 days pregnant rats (open squares) and 17-21 days pregnant rats (closed squares).

From Figures 3, 4 and 5, it is evident that pregnancy modifies the response of the rat portal

Table 1 Venous activity changes during pregnancy

	Nonpreg	*17-21 days preg*	*Significance*
Amplitude of contraction (mN/mg)	0.67 ± 0.06	0.53 ± 0.07	$P < 0.05$
Number of contractions/10 min	39.57 ± 2.67	36.0 ± 4.96	NS
Total contractile activity mN/mg	87.6 ± 7.9	68.1 ± 9.5	$P < 0.05$

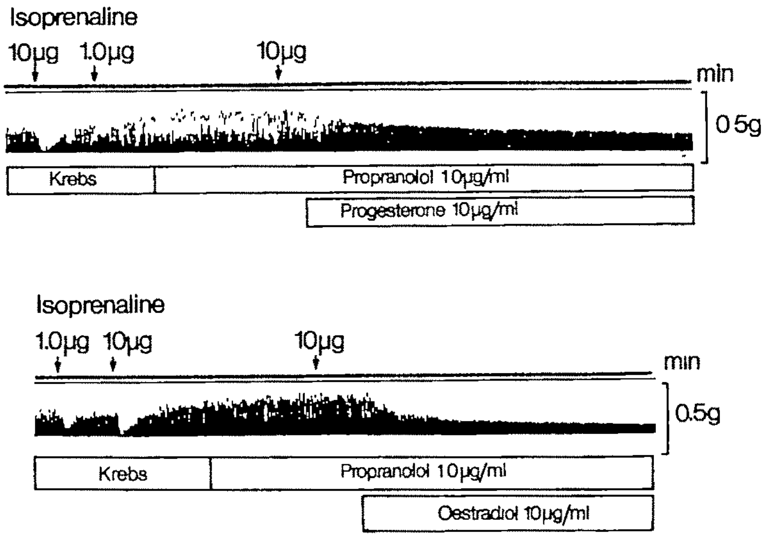

Figure 6 The spontaneous mechanical activity of a rat portal vein when 10 µg/ml progesterone (upper trace) or 10 µg/ml oestradiol (lower trace) was used after β-receptor blockade with propranolol (1 µg/ml). Contraction and time calibrations are shown.

vein to the steroids. The sensitivity to progesterone was similar in the non-pregnant and 7 days pregnant groups. The 10-14 days pregnant group showed slight hypersensitivity while the final 17-21 days pregnant group was hyposensitive. The maximum sensitivity to oestradiol occurred in the 7 days pregnant group.

Initial levels of spontaneous activity As well as changing the sensitivity to the steroids pregnancy seemed to have an influence on the initial amplitude of activity when normal Krebs was used as a perfusate. Table 1 shows the amplitude of contraction, number of contractions and the total contractile activity in the non-pregnant and 17-21 days pregnant groups. The average amplitude of contraction and the total contractile activity were significantly lower in the pregnant than non-pregnant group ($P < 0.05$). There was no significant change in the number of contractions/10 minutes ($P < 0.25$).

Thus pregnancy seems to modify both the spontaneous activity of the rat portal vein and the sensitivity of this preparation to progesterone and oestradiol-17-β.

The effects of β-adrenoceptor blockade

Figure 6 shows two records of the spontaneous mechanical activity of two rat portal veins. The upper trace shows the activity when progesterone (10 µg/ml) was used. At the beginning of the experiment when normal Krebs solution was being used, isoprenaline (10 µg and 1 µg) caused inhibition of the spontaneous activity by stimulation of the β-adrenoceptors. After 30 min of perfusion with propranolol (1 µg/ml) these receptors were shown to be blocked because isoprenaline (10 µg) had no effect. When progesterone (10 µg/ml) was used its inhibitory effect on the amplitude of contraction was still evident. The lower trace shows a similar record when oestradiol (10 µg/ml) was used after β-blockade. Inhibition of the amplitude of contraction was still present. The same result occurred in all six experiments which were carried out.

The effect of hydrocortisone

The spontaneous mechanical activity of a rat portal vein before and after exposure to hydrocortisone (10 µg/ml) was also measured. Unlike oestradiol or progesterone, hydrocortisone caused no apparent inhibition of the muscle. This result was found in each of the three experiments which were carried out.

Discussion

The rat portal vein carries blood from the intestine, stomach and spleen to the liver. Thus the blood in the vein has already been through one microcirculation and must be at a much reduced hydrostatic pressure. Normally the movement of blood in the peripheral venous system is helped either by pressure exerted externally during contraction of surrounding skeletal muscles which forces blood through a one way system of valves, or by negative pressure from the abdomen. Neither of these mechanisms can be applied to the hepatic portal vein because it is surrounded by fatty tissue of the abdomen and hence cannot have an external muscle pump. Secondly, it runs between two sets of capillaries and it would seem unlikely that negative pressure in the inferior vena cava would be transmitted through the liver to the portal vein. One possible mechanism for the movement of blood along the vein could be the inherent spontaneous activity of the smooth muscle wall in the vessel. This spontaneous contractile activity could be functioning as an intrinsic muscle pump. By inhibiting this spontaneous activity the blood in the vessel is left only with the driving force of hydrostatic pressure transmitted through the intestinal capillaries. The evidence presented in the present experiments demonstrates that the steroids oestradiol-17-β and progesterone are capable of inhibiting the spontaneous activity of the longitudinally arranged muscle fibres in the rat portal vein. In conjunction with an inhibition of the circularly oriented fibres stasis of blood would therefore result from *in vivo* administration of oestradiol or progesterone. The stasis of blood flow in conjunction with the steroid-induced hypercoagulability (Phillips, 1963) is proposed as a possible cause of the cases of mesenteric venous thrombosis described by Berjain (1969) and Hurwitz *et al.* (1970).

Natural sources of progesterone and oestradiol are the ovary and the placenta. During pregnancy large amounts are produced, particularly by the placenta, and the systemic blood concentration of the steroids is higher. The comparisons of portal veins taken from groups of rats at different stages of gestation was an attempt to see if this naturally elevated blood steroid level would have an effect on the spontaneous activity of the vein. The results showed that there was a significant difference in the spontaneous activity between the portal veins from the non-pregnant group and the veins from the 17-21 days pregnant group. Kuriyama & Csapo (1961) showed that strips of rabbit uterine muscle taken at different stages of gestation had markedly different mechanical properties. At the beginning of the pregnancy the muscle strip was spontaneously active and when stimulated all the strip contracted. At the end of the pregnancy this situation was reversed. The muscle was mechanically and electrically quiescent and there was no propagation of induced contraction down the tissue. The present experiments indicate that a similar reduction in the spontaneous activity in the portal vein of the rat occurs during pregnancy. Kuriyama & Csapo (1961) implicated progesterone as the cause of the uterine quiescence. The possibility that the steroids were involved in the portal venous inhibition seems likely, as they caused this effect *in vitro*.

The present results show that a change in the activity of the portal vein occurs at the end of a pregnancy. The systemic blood levels of oestrogens at this time have been shown to be less than 1 ng/ml (Yoshinaga, Hawkins & Stocker, 1969). Progestins have however been measured at 0.13 μg/ml (Hasimoto, Henricks, Anderson & Melampy, 1968). Although these concentrations have not been shown to be effective in the present acute experiments the possibility exists that chronic exposure to the circulating steroid (particularly progesterone) is effective in producing the recorded changes in activity between the non-pregnant and the 17-21 days pregnant group. Sensitivity of the veins to added oestradiol and progesterone was greatest with vessels removed at the beginning of pregnancy. The circulating steroids may have caused accommodation of the venous muscle and hence as pregnancy progressed the action of the added steroids becomes smaller. Oestradiol throughout was more effective than progesterone and instead of a single inhibitory effect it displayed a biphasic effect. At low concentrations, oestradiol appeared to stimulate the muscle to greater activity, but at all higher concentrations caused the inhibition and acceleration of the vessel. Normally, the steroids caused the inhibition of the amplitude of contraction, but acceleration of the frequency of contraction. If these steroids had the same effect on the venous muscle as progesterone has on the uterine strip (Kuriyama & Csapo, 1961) then the increase in frequency could be due to a decrease in the propagation of contraction through the tissue. Thus, instead of the venous muscle all contracting with a large amplitude in response to one unstable pacemaker cell, there are many such pacemaker cells producing small but frequent contractions. The increased frequency of contractions would then only be a stage towards the cessation of activity. This pattern was in fact found when oestradiol was applied to portal veins from rats which were 7 days pregnant (Figures 6, 7 and 8). Axelsson, Wahlström, Johansson & Jonsson (1967) found that a similar pattern occurred when the rat portal vein was artificially hyperpolarized using low potassium solutions as a bathing medium. The muscle was found to have a transient increase in frequency but decrease in amplitude of contractions. After 10 min all activity stopped. Oestradiol and progesterone were found by many workers to cause hyperpolarization of uterine muscle (Marshall, 1959; Goto & Csapo, 1959; Kuriyama & Csapo, 1961). In view of the findings of Axelsson *et al.* (1967) it seems likely that the mechanism of action of the steroids on the venous muscle is at least partly by hyperpolarization of the cell membrane. Oestradiol causes myometrial hyperpolarization and moves the membrane potential into the 'firing range' for spontaneous action potentials. The membrane potential of venous muscle is however already in its 'firing range'. Thus a low dose of oestradiol (0.1 μg/ml) must have moved the membrane potential into a more excitable area of this 'firing range' whereas the higher concentrations (1.0 μg/ml and 10 μg/ml) hyperpolarized the tissue out of this range completely. Progesterone causes uterine hyperpolarization and moves the membrane potential beyond the 'firing range'. Its action on venous muscle would be similar.

Shabanah, Toth, Carassavas & Maughan (1968) have suggested that the adrenoceptors of the uterus play a large part in the mediation of the effects of oestradiol and progesterone. Progesterone was proposed to act via the β-receptors to cause uterine quiescence during the pregnancy. β-receptor stimulation in the venous muscle caused much the same effects as the steroids. However, the present evidence shows that the two pathways are completely separate and that progesterone or oestradiol do not act via the β-receptors in venous muscle.

Thus these hormones have been shown to inhibit the activity of the muscle component of the vessel wall. Connective tissues have also been shown to be affected. Buckingham, Seldon & Danforth (1962) showed that connective tissues in the cervix were weakened during pregnancy and postulated that 'probably all connective tissue structures, not only the cervix undergo alteration and loss of tensile strength during pregnancy'. Later Danforth, Manalo-Estrella & Buckingham, (1964) found that the steroids oestradiol and progesterone did produce a similar loss of tensile strength in the connective tissue structures of many arteries and veins.

The steroid-induced weakness in the vein wall and the steroid inhibition of the smooth muscle must make the vessel more liable to dilate. In such a situation, an increase in venous blood pressure (due to the occlusion effects of an enlarging uterus) would be very effective in producing blood stasis and varicose veins. Thrombosis could quickly follow as the steroids have also been shown to produce a state of hypercoagulability in blood (Mishra & Mishra, 1969).

Thus the inhibitory action of oestradiol-17-β and progesterone on venous muscle has been demonstrated. A similar inhibition was found during the course of a pregnancy. It is postulated that these steroids act on the venous muscle in the same way as the myometrium and that the prediction of Kilbourne (1933) that there is a general loss of smooth muscle tone in all structures of the pregnant body may be correct, and may be due to the increased amount of circulating oestrogens and progesterone.

I wish to thank Professor I.C. Roddie and Dr B.N. Barwin for their help and advice. Also the technical staff of the Queens University Physiology Department for their skilled assistance. This work was carried out during the tenure of an M.R.C. Training Award.

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TRYPTAMINES AND SOME OTHER SUBSTANCES AFFECTING WAKING AND SLEEP IN FOWLS

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1 Adult fowls (*Gallus domesticus*) with cannulae chronically implanted in the IIIrd cerebral ventricle and various other sites of the brain, received infusions or injections of tryptamines and catecholamines into the brain; effects of and interactions between these substances on behaviour, electrocortical activity and body temperature were studied. Reserpine-induced arousal, was investigated in young and adult fowls.

2 Tryptamine and α-methyltryptamine, given intraventricularly or into the hypothalamus of intact fowls evoked behavioural and bilateral electrocortical arousal, postural changes, elevation of body temperature and tachypnoea; behavioural and bilateral electrocortical arousal were obtained with infusions into the mesencephalon. Ipsilateral electrocortical arousal only, resulted from infusion of α-methyltryptamine into the hypothalamus or mesencephalon of fowl encéphale isolé preparations. The above effects in intact fowls were reduced or replaced by sleep following administration of noradrenaline or α-methylnoradrenaline into the IIIrd ventricle or hypothalamus Pretreatment of intact fowls with an amine oxidase inhibitor surprisingly attenuated or reversed the excitant effects of intraventricular tryptamine.

3 5-Hydroxytryptamine (hydrogen maleinate, creatinine sulphate or oxalate) given intraventricularly or infused into the hypothalamus, elevated body temperature; tachypnoea and postural changes developed at some stage during the elevation of body temperature. Sleep also was induced, although with the oxalate this was succeeded by marked arousal.

4 Behavioural and electrocortical sleep induced by 5-hydroxytryptamine infused into the hypothalamus were replaced by arousal on infusing tryptamine into the hypothalamus, and *vice versa.*

5 Dexamphetamine infused into the hypothalamus induced drowsiness or sleep which even reversed arousal elicited by systemically administered dexamphetamine.

6 Reserpine-induced arousal was achieved in young and adult fowls pretreated with mebanazine; this arousal was attenuated or replaced by sleep following intraventricular noradrenaline or dopamine but not by 5-hydroxytryptamine nor by noradrenaline or dopamine applied to the hypothalamus. Prenylamine also induced arousal following pretreatment of chicks with mebanazine.

Introduction

Tryptamine, α-methyltryptamine and dexamphetamine given systemically elicit behavioural and electrocortical arousal (Dewhurst & Marley, 1965a, b), and elevate body temperature and oxygen consumption (Allen & Marley, 1967) in fowls. Arousal was assumed to be due to an action on the brain, since intravenous injection of these amines also evoked behavioural and electrocortical arousal in chicken encéphale isolé preparations (Marley & Stephenson, 1971). Brief arousal, followed by longer-lasting sleep, was obtained with intravenous 5-hydroxytryptamine (Hehman, Vonderahe & Peters, 1961; Dewhurst & Marley, 1965b).

Present experiments were undertaken in adult fowls to determine whether intraventricular injection or intracerebral infusion of these amines elicited similar effects to intravenous administration; their site of action was also investigated by infusing the amines into various brain areas. Effects obtained with systemically administered tryptamine, α-methyltryptamine, and 5-hydroxytryptamine, which had been ascribed to central actions, were indeed elicited by their application to the hypothalamus and mesencephalon although this was not the case for amphetamine. A brief account of this work has been published (Marley & Nisticò, 1971).

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Behavioural arousal resembling that obtained with amphetamine, has been induced by reserpine in a number of species following pretreatment with an amine oxidase inhibitor (Brodie, Pletscher & Shore, 1956; Chessin, Kramer & Scott, 1957; Graeff, Leme & Silva, 1965; Bueno, Pscheidt & Himwich, 1968). The syndrome, not described hitherto in fowls, was elicited by prenylamine as well as by reserpine. Arousal under these circumstances has been attributed in mammals to release by reserpine within the brain of noradrenaline and/or dopamine (Graeff *et al.*, 1965) or 5-hydroxytryptamine (Chessin *et al.*, 1957). Dopamine, noradrenaline or 5-hydroxytryptamine were therefore given into the brain of fowls, roused by reserpine after mebanazine, to determine whether their administration enhanced or mitigated the syndrome.

Methods

Rhode Island Red fowls of 1.5-2.0 kg and housed at 20°-25°C were used as were 10-16 day old pullets kept in a thermostatically controlled cage at 29°-31°C, ambient temperatures within their thermoneutral ranges.

For operative procedures under halothane anaesthesia and experimental methods, see Marley & Nisticò (1972). Cannulae were implanted stereotactically into the brain under aseptic conditions. Brain coordinates for stereotactic implantation of cannulae not given in Marley & Nisticò (1972), include telencephalon A + 8.0, L + 8.0, V + 9.0; paleostriatum augmentatum A + 9.0, L = 2.5, V + 7.5; mesencephalon A + 4.0, L = 3, V + 3.5 and A + 2.2, L = 1, V + 3.0 (from atlas of van Tienhoven & Juhász, 1962). Fowls were not tested until at least a week after recovery from operative procedures, and thereafter at intervals of at least a week. Cannulae positions were subsequently located by histological examination of the brain. Elevation of body temperature with the tryptamines appeared to have been more readily elicited in fowls with the cannula tip found to be in the anterior rather than in the posterior hypothalamus.

Drugs used were hydrochlorides of *p*-chlorophenylalanine methyl ester, dopamine, (-)-α-methylnoradrenaline, nialamide, (+)-α-methyltryptamine and tryptamine; the oxalate, creatinine sulphate and hydrogen maleinate salts of 5-hydroxytryptamine; dexamphetamine sulphate; mebanazine oxalate, methysergide bimaleate; sodium oxalate, disodium maleinate and creatinine sulphate; prenylamine gluconate (Segontin gluconate 5%, Hoechst); reserpine was dissolved in glacial acetic acid and buffered with 8% NaOH. (−)-Noradrenaline base was dissolved immediately before use in equimolar hydrochloric acid.

Results

Tryptamine

Intraventricular Infusion of tryptamine, 0.5 and 1.0 μmol, evoked intense behavioural and electrocortical alerting within 5 to 10 min in 5 of 7 fowls tested. Rapid jerking movements of the head also developed which periodically escalated into repetitive preening of body feathers. Vocalization, uncommon in adult fowls isolated in the observation box, became frequent, as did pecking. Some 15-20 min after tryptamine the wings were abducted at an angle of about 30° from the trunk, the primary wing feathers diverging and emerging from behind the secondary wing feathers. The tail was extended, and the back became horizontal or even sloped downwards from the tail to the head, replacing the normal slope of the spine. (These changes in the alignment of body and wings are described hereafter as postural changes.) Electrocortical, behavioural and postural changes persisted for 30 to 120 min, depending on dose. Tachypnoea developed almost immediately after tryptamine, respiratory rate increasing from control values of 20 to 30/min up to 100 to 160/min and slowly subsiding over the ensuing 60 to 90 minutes. Increases of body temperature of between 0.5° and 2.0°C, maximum about 60 min after infusion, were noted in 6 of the 7 fowls. When tryptamine, 0.1-0.3 μmol, was infused into the *hypothalamus* of normally alert fowls similar intense arousal and postural changes were produced, except that their onset was preceded by sleep for 5 to 10 minutes. *Mesencephalic* infusion of tryptamine, 0.2 and 0.3 μmol (2 normally alert fowls), also evoked intense electrocortical and behavioural alerting following sedation for 5-10 min, but the postural changes were absent. Infusions into the *paleostriatum augmentatum* of tryptamine, 0.3 μmol (3 fowls) did not affect behaviour, temperature and electrocortical activity.

Antagonism between tryptamine and noradrenaline Effects of tryptamine given into the IIIrd ventricle or hypothalamus were attenuated or antagonized by prior or subsequent infusion of noradrenaline into the hypothalamus. Thus with a fowl in which intraventricular tryptamine 1.0 μmol evoked typical behavioural, electro-

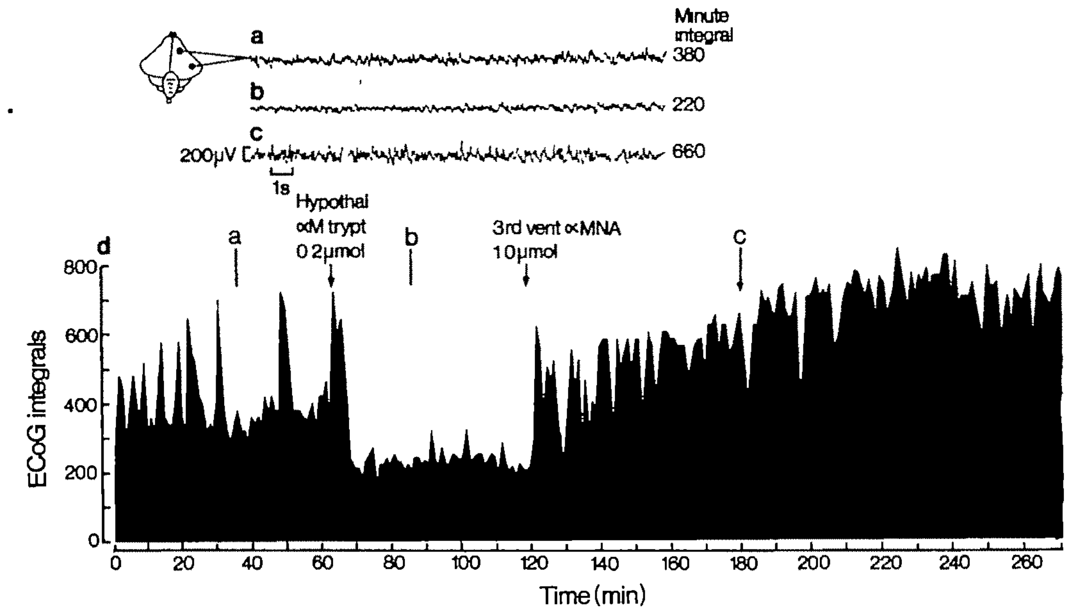

Figure 1 Antagonistic effect in an adult fowl of α-methylnoradrenaline (αMNA) on electrocortical arousal induced by α-methyltryptamine (αMtrypt). (a) Control drowsy electrocortical activity. (b) Electrocortical alert activity following α-methyltryptamine (αMtrypt) (0.2 μmol) infused into the hypothalamus and accompanied by behavioural arousal. (c) Sleep electrocortical activity accompanying behavioural sleep and following α-methylnoradrenaline (1.0 μmol) injected intraventricularly. (d) Integrals corresponding to electrocortical activity showing decrease with electrocortical arousal and increase with electrocortical sleep.

cortical, respiratory and postural effects and increase in body temperature from 40.8°C to 41.5°C, noradrenaline, 0.1 μmol infused into the hypothalamus 45 min later, induced sleep for 2 h, abolished tachypnoea and within the ensuing 30 min, lowered body temperature to 39°C. In another fowl in which noradrenaline (0.1 μmol into the IIIrd ventricle) induced profound behavioural and electrocortical sleep, tryptamine (0.2 μmol) infused into the hypothalamus 60 min later, when the fowl was still deeply asleep, evoked its characteristic effects although these were attenuated. Thus behavioural and electrocortical arousal ensued for 20 min before the fowl reverted again to sleep; respiratory rate increased from 9/min to 60-80/min and there was slight wing abduction.

Monoamine oxidase inhibition The behavioural effects of intraventricular tryptamine were unexpectedly modified, and only the temperature changes potentiated by prior amine oxidase inhibition. Thus, in a normally alert fowl, after nialamide (100 μmol/kg, i.p. 16 h and 1 h previously) tryptamine, 0.5 μmol, instead of producing intense arousal, induced behavioural and electrocortical sleep within 5 min, effects which lasted 3 hours. In two fowls pretreated with mebanazine (100 μmol/kg, i.p. 16 h and 1 h previously in one and 40 h, 16 h and 1 h previously in the other) intraventricular tryptamine, 0.5 μmol, lacked effect on behaviour although previously eliciting intense arousal in the absence of amine oxidase inhibition; body temperature was increased by 1° and 1.5°C for 6 h and 5 h respectively compared with 1 to 2 h before mebanazine.

α-Methyltryptamine in intact fowls

Injection of α-methyltryptamine (0.5 and 1.0 μmol) into the *IIIrd ventricle* (3 fowls) evoked behavioural, electrocortical, respiratory and postural changes within 5-10 min, similar to those after intraventricular tryptamine but lasting up to 4 hours. Infused into the *hypothalamus* (3 fowls) and *mesencephalon* (2 fowls), α-methyltryptamine (0.2 to 0.4 μmol) elicited behavioural and electrocortical arousal (Figure 1) within 3 to 5 min similar to that observed after tryptamine but of longer duration (2 to 3 h); infusions into the *telencephalon* lacked effects.

Antagonism between α-methyltryptamine and noradrenaline or α-methylnoradrenaline The effects of intrahypothalamic α-methyltryptamine

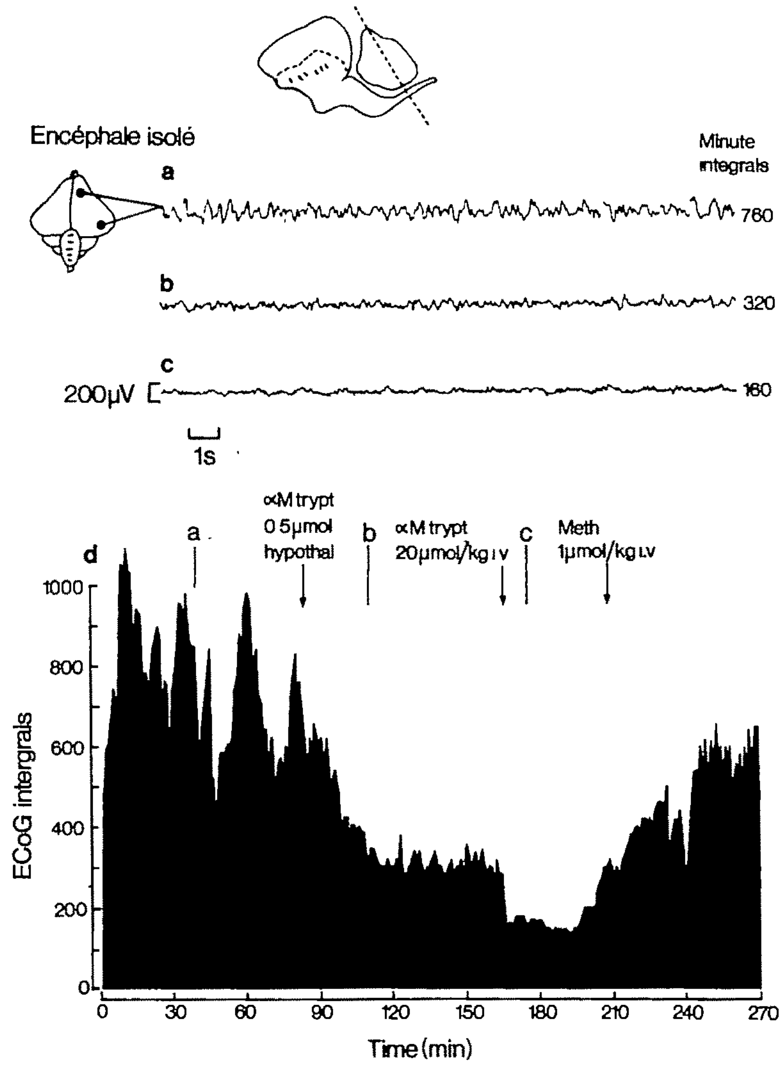

Figure 2 Electrocortical activity in a fowl encéphale isolé preparation (dotted line indicates location of transection) showing electrocortical arousal with α-methyltryptamine (αMtrypt) infused into the hypothalamus (compare b, with control sleep activity in a), with intensification of arousal by intravenous α-methyltryptamine (c). (d) Integrals of electrocortical activity showing reduction of these by α-methyltryptamine and antagonism by methysergide (Meth).

were antagonized by α-methylnoradrenaline (1.0 µmol) given into the IIIrd ventricle, as shown for electrocortical activity in Figure 1. In another fowl, behavioural and electrocortical sleep induced by intraventricular noradrenaline (1.0 µmol), were reversed to behavioural and electrocortical arousal lasting about 2 h following α-methyltryptamine (0.4 µmol) infused into the hypothalamus.

α-Methyltryptamine in fowl encéphale isolé preparations

The effects of α-methyltryptamine were also studied in fowl encéphale isolé preparations which in the absence of drug administration, exhibit sustained behavioural and electrocortical sleep (Marley & Stephenson, 1971). The infusion cannula was implanted into the hypothalamus in 7 fowls, into the mesencephalon in 2, and immediately posterior to the mesencephalon in 2 other fowls.

Unilateral electrocortical desynchronization developing over 20 min, but without behavioural arousal, was elicited by α-methyltryptamine (0.5 µmol) infused into the ipsilateral hypo-

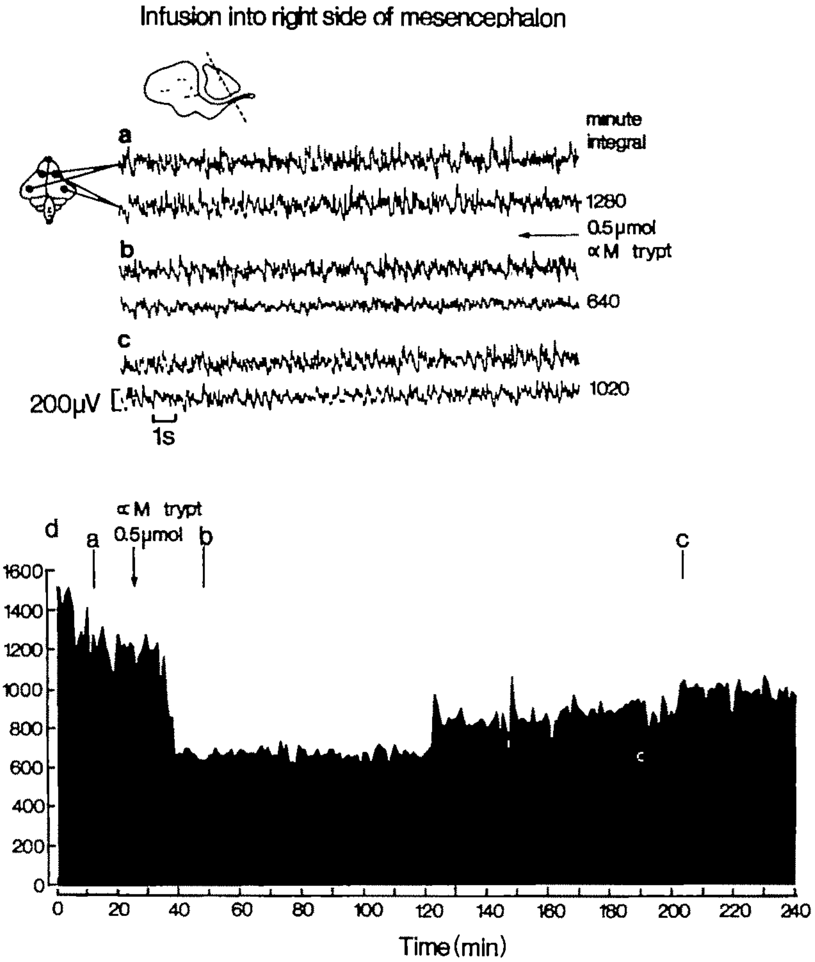

Figure 3 Records of electrocortical activity from a fowl encéphale isolé preparation (dotted line indicates transection) illustrating unilateral electrocortical arousal following α-methyltryptamine (αMtrypt) infused into the mesencephalon. (a) Control sleep electrocortical activity. (b) Electrocortical arousal evident in recording taken from the right cerebral hemisphere 22 min after α-methyltryptamine (0.5 μmol) infused into the ipsilateral mesencephalon, together with halving of electrocortical integrals. (c) Return of sleep electrocortical activity 180 min after α-methyltryptamine with corresponding increase in electrocortical integrals. (d) Integrals corresponding to electrocortical activity.

thalamus (Figure 2b). Subsequent intravenous injection of α-methyltryptamine (20 μmol/kg) intensified electrocortical desynchronization (Figure 2c, d) and extended it to both cerebral hemispheres. These effects, which were accompanied by behavioural arousal, were antagonized by methysergide (1.0 μmol/kg, i.v.), as evident for electrocortical activity from the increase in electrocortical integrals (Figure 2d). Ipsilateral electrocortical desynchronization, developing over 15 min, was much less marked with infusion of α-methyltryptamine (0.5 μmol) into the mesencephalon (Figure 3), and was not elicited with more posterior infusions.

5-hydroxytryptamine

Three forms of 5-hydroxytryptamine and the respective salts were tested, since Roberts & Straughan (1967) reported that the effects of iontophoretically applied 5-hydroxytryptamine varied according to the salt used.

5-hydroxytryptamine oxalate and sodium oxalate, each 0.2 and 0.3 μmol, produced identical effects when infused into the *hypothalamus* (4 fowls). Thus within 5 to 10 min of infusion, the fowl became more behaviourally and electrocortically alert with head shaking, wing abduction, vocaliza-

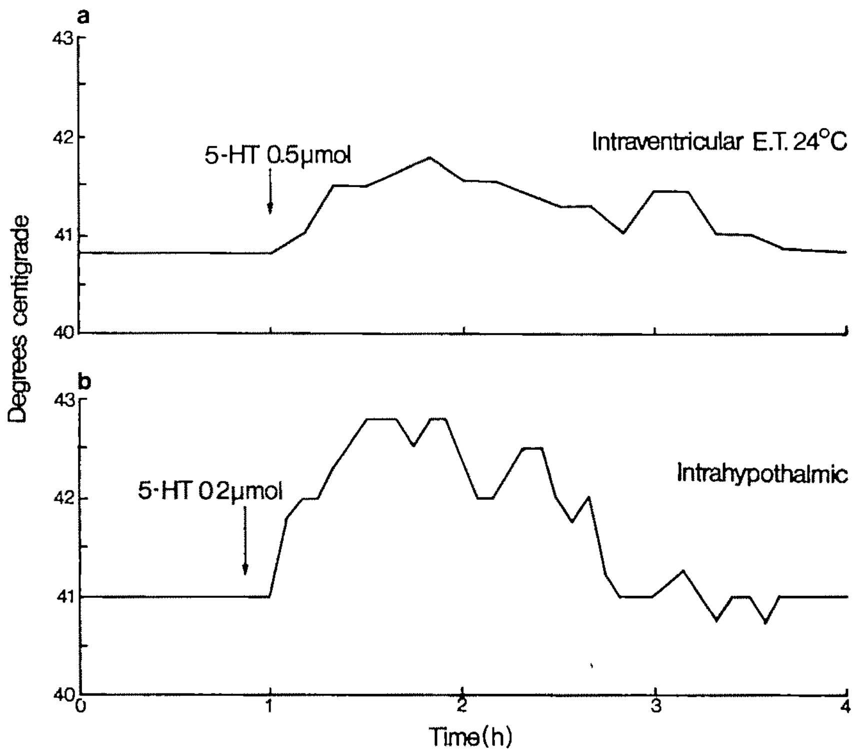

Figure 4 Body temperature responses in same fowl to 5-hydroxytryptamine (5-HT) hydrogen maleinate given (a) intraventricularly, and (b) a week later, into the hypothalamus, environmental temperature (E.T.) was 24° C for both experiments Note, more intense elevation of body temperature with smaller dose of 5-hydroxytryptamine into the hypothalamus.

tion, 'escape' responses and tachypnoea (60-180/minute). This state lasted 20 to 30 min and was followed by drowsiness for up to 1 hour. Body temperature rose by up to 2.0°C. *Intraventricular* injection of 5-hydroxytryptamine 0.3 to 0.5 µmol, elicited initial drowsiness or sleep for 5 to 30 min (3 normally alert fowls), followed by behavioural and electrocortical arousal for 30 min, together with tachypnoea (60-120/min), vocalization, preening, head-shaking and abduction of the wings from the trunk; body temperature was not significantly altered.

5-hydroxytryptamine hydrogen maleinate. After *hypothalamic* infusion of 5-hydroxytryptamine, 0.1 and 0.2 µmol (3 alert fowls), body temperature increases ranged from 0.75° to 2.8°C, reaching a peak after about 20 min with recovery in 2 to 3 hours (Figure 4b). After about 5 min, 2 of the fowls squatted asleep for 30 min but the third remained alert; once sleep abated, tachypnoea and wing abduction developed. Equimolar doses of disodium maleinate lacked effect when given by this route. In contrast after *intraventricular* disodium maleinate 1.0 µmol, body temperature increased 0.8° and 1.3°C respectively (2 fowls), together with tachypnoea (124/min and 110/min respectively), wing abduction, excitement and escape responses; body temperature and excitation subsided, after 35 minutes. In 4 normally alert fowls, given *intraventricular* 5-hydroxytryptamine (0.5 and 1.0 µmol), body temperature increased over 30 min up to 1°C with recovery in 2 to 3 h (Figure 4a); sleep developed within 5 min and lasted 30 min with tachypnoea and wing abduction appearing once the fowls were alert.

5-hydroxytryptamine creatinine sulphate (0.1 and 0.2 µmol) infused into the *hypothalamus* of 2 fowls evoked sleep, commencing after 5 min and lasting 2 to 3 h, although body temperature was unaltered; sleep was also induced by equimolar infusions of creatinine sulphate (2 fowls) into the hypothalamus. *Intraventricular* 5-hydroxytryptamine, 0.5 µmol, induced sleep but lacked temperature effects. 5-hydroxytryptamine, 1.0 µmol, induced sleep within 5-10 min which lasted

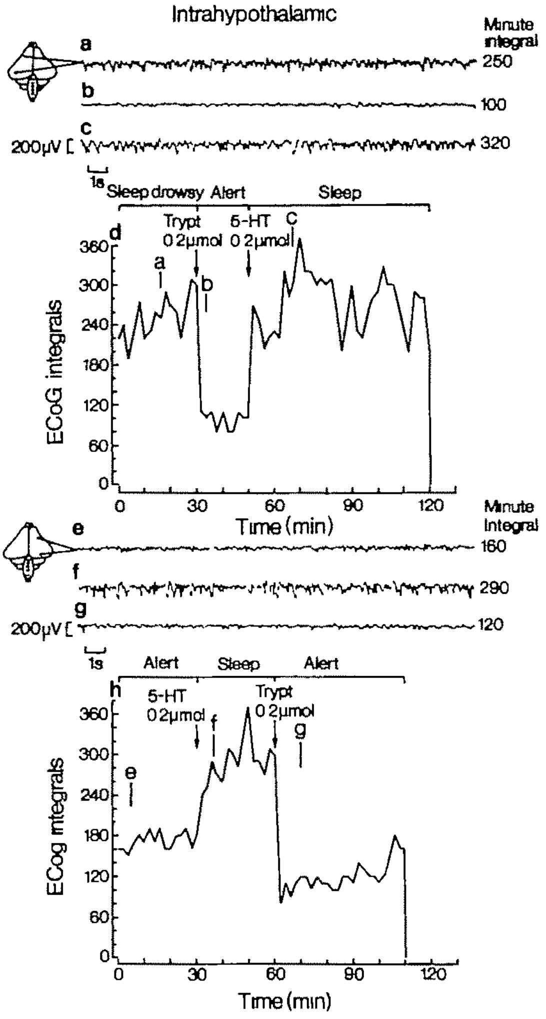

Figure 5 Records of electrocortical activity with corresponding integrals of electrocortical activity in two fowls (a-d and e-h, respectively) to demonstrate that arousal evoked by tryptamine (Trypt) was abolished and replaced with sleep induced by 5-hydroxytryptamine (5-HT), and *vice versa*. (a) Control sleep electrocortical activity. (b) Alert electrocortical activity 4 min after tryptamine, 0.2 μmol, infused into the hypothalamus, accompanying behavioural arousal. (c) Sleep-like electrocortical activity 19 min after 5-hydroxytryptamine, 0.2 μmol, infused into the hypothalamus and associated with behavioural sleep. (d) Electrocortical integrals demonstrating marked reduction during tryptamine-evoked arousal and subsequent rapid and substantial increase following 5-hydroxytryptamine (e) Control alert electrocortical activity (f) Sleep-like electrocortical activity 6 min after 5-hydroxytryptamine, 0.2 μmol, infused into the hypothalamus, accompanying behavioural sleep. (g) Alert electrocortical activity 10 min after tryptamine, 0.2 μmol, infused into the hypothalamus and associated with behavioural arousal. (h) Electrocortical integrals exhibiting marked increase during 5-hydroxytryptamine-induced sleep and subsequent rapid and substantial decrease following tryptamine.

55 min; body temperature was elevated 1.0°C with recovery after 160 min; heat-loss mechanisms were activated since wing abduction developed after 105 min, lasting 50 min, accompanied by tachypnoea (respiratory rate 42-86/min) for 20 minutes.

Antagonism between tryptamine and 5-hydroxytryptamine. Since tryptamine and 5-hydroxytryptamine had opposite effects on behaviour and electrocortical activity, they were conceivably acting on different receptors or neuronal systems. Experiments were therefore undertaken to ascertain whether, in the same fowl, arousal due to tryptamine was abolished and replaced by sleep following infusion of 5-hydroxytryptamine hydrogen maleinate, into the hypothalamus, and *vice versa*. The first of the 3 fowls tested, initially slept or drowsed (electrocortical activity and integrals, Figure 5a, d). Tryptamine, 0.2 μmol, infused into the hypothalamus then evoked electrocortical arousal within 4 min (Figure 5b), together with reduction of electrocortical integrals from between 190-310/min to about 80-100/min (Figure 5d); this was accompanied by behavioural arousal, wing abduction, vocalization, tachypnoea and pecking. 5-Hydroxytryptamine, 0.2 μmol, infused into the hypothalamus 20 min subsequently (a time when behavioural and electrocortical arousal would have persisted a further 20-40 min, see Figure 5h), replaced the alert state within 5 min by one of sleep, pecking and vocalization abating. The electrocortical record (Figure 5c) and increase in integrals (Figure 5d) represent changes 18 min after 5-hydroxytryptamine. In contrast, the second fowl was initially electrocortically (Figure 5e, h) and behaviourally alert but 5-hydroxytryptamine, 0.2 μmol, infused into the hypothalamus induced electrocortical sleep within 6 min (Figure 5f), electrocortical integrals increasing from between 150-190/min, to a peak of 370/min (Figure 5h), behavioural sleep also developed. Thirty minutes later, tryptamine, 0.2 μmol, infused into the hypothalamus elicited electrocortical arousal (Figure 5g) and diminished electrocortical integrals to approximately 100/min (Figure 5h); sleep was replaced by behavioural arousal accompanied by pecking, vocalization and wing abduction. Similar results were obtained in a third fowl tested.

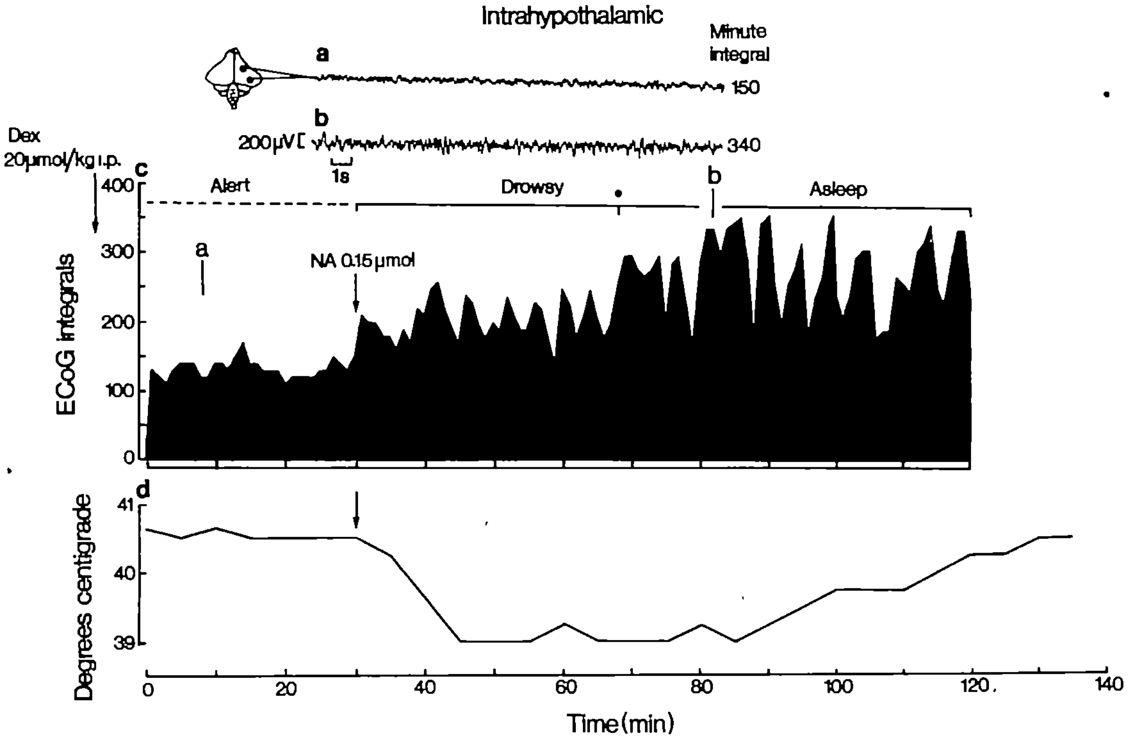

Figure 6 Antagonistic effects in an adult fowl of noradrenaline (NA) on electrocortical arousal induced by dexamphetamine (Dex). (a) Control electrocortical activity after dexamphetamine (20 μmol/kg, i.p.) given 40 min previously. (b) Electrocortical sleep activity 55 min after noradrenaline (0.15 nmol) infused into the hypothalamus. (c) Histogram of integrated electrocortical activity showing progressive increase following intrahypothalamic infusion of noradrenaline, as the fowl becomes drowsy and then asleep. (d) Fall of body temperature up to 1.5° C following noradrenaline.

Dexamphetamine

Dexamphetamine, 0.2 to 0.5 μmol, infused into the *hypothalamus* (3 fowls), evoked behavioural and electrocortical drowsiness or sleep of 1 to 2 h duration. Interestingly, behavioural and electrocortical arousal elicited in a fowl by intraperitoneal injection of dexamphetamine 20 μmol/kg (a dose sufficient to sustain arousal for 60 min) was replaced 10 min later by behavioural and electrocortical sleep for 55 min, almost immediately following dexamphetamine, 0.8 μmol, infused into the hypothalamus.

Antagonism between dexamphetamine and either noradrenaline or α-methylnoradrenaline Two fowls were tested, intraperitoneal dexamphetamine (20 μmol/kg) being given to induce behavioural and electrocortical arousal. Noradrenaline (0.15 μmol) in one fowl and α-methylnoradrenaline (0.1 μmol) in the other, were infused respectively 40 and 30 min later into the hypothalamus. Both fowls became drowsy and then profoundly asleep, sleep lasting at least 3 h and the alert electrocortical activity (Figure 6a) changing to large amplitude, slow frequency potentials (Figure 6b) electrocortical integrals increasing two to threefold (Figure 6c) and body temperature falling 1.4° C (Figure 6d).

Arousal induced by reserpine following mebanazine

Adult fowls After pretreatment with mebanazine (100 μmol/kg, i.m. 18 h and 2 h previously), reserpine (20 μmol/kg, i.m.) was given to thirteen fowls. In ten of these, reserpine induced behavioural and electrocortical drowsiness or sleep lasting 45-120 min followed by more than 6 h of intense arousal (Figure 7a-c). During the drowsy period, the fowls stood immobile with lowered wings and flexed tail whereas during the ensuing alert state, the fowls evinced marked locomotor activity, pecked the walls and floor of the

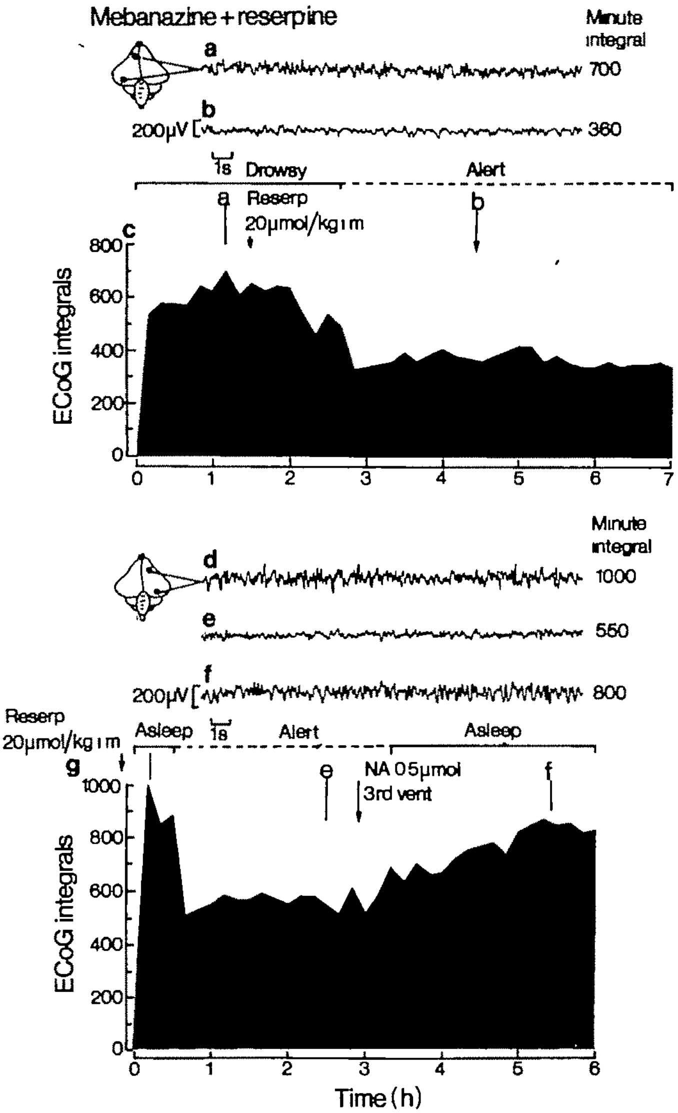

Figure 7 Records of electrocortical activity with corresponding integrals of electrocortical activity in two fowls (a-c and d-g respectively) pretreated with mebanazine (100 μmol/kg, i.m. 18 h and 2 h previously). (a) Control sleep electrocortical activity. (b) Alert electrocortical activity 3 h after reserpine (Reserp) (20 μmol/kg, i.m.). (c) Electrocortical integrals showing marked reduction during reserpine-induced arousal and persisting for 5.5 h when the experiment was terminated. (d) Control sleep electrocortical activity in another fowl pretreated with mebanazine as above and followed by reserpine (20 μmol/kg, i.m.) (e) Alert electrocortical activity during reserpine-induced arousal. (f) Sleep electrocortical activity associated with behavioural sleep 2.5 h following noradrenaline (NA 0.5 μmol) injected into the third ventricle. (g) Electrocortical integrals showing marked reduction during reserpine-induced arousal, followed by increase associated with sleep evoked by intraventricular noradrenaline.

observation box and vocalized, unusual in the test situation; the wings were abducted about 60° from the trunk and respiratory rate rose to 200/minute. This alert phase was absent in the remaining 3 fowls. Body temperature changes (±0.75°C) gave no indication as to which amine was involved in the syndrome, and indeed occurred whether reserpine induced arousal or not.

Noradrenaline administration In 3 fowls with reserpine-induced arousal, intraventricular noradrenaline (0.5 or 1.0 μmol) replaced within 10 to

15 min the intensely alert behavioural and electrocortical activity by electrocortical (Figure 7d-g) and behavioural sleep lasting 4 h or more; the fowls slept squatting or standing. Tachypnoea (up to 200/min) which had developed, disappeared during sleep induced by noradrenaline. In contrast, noradrenaline (0 1 or 0.2 μmol) infused into the hypothalamus of 3 fowls did not diminish arousal induced by reserpine. The long lasting fall of body temperature of 1.5°-2 0°C which follows intraventricular or intrahypothalamic administration of noradrenaline in adult fowls after mebanazine (Marley & Nisticò, 1972) was not observed, body temperature varying ±0.5°C, except in one fowl that died within 24 h of the experiment, in which body temperature rose from 41° to 45 25°C.

Dopamine administration Three fowls were pretreated with mebanazine, 100 μmol/kg, i.m 18 and 2 h prior to reserpine 20 μmol/kg, i.m. Once behavioural arousal had developed and was intense, dopamine (0.5, 1.0, 2.0 μmol respectively) was given into the IIIrd ventricle Sedation was apparent in 3 to 10 min, followed by behavioural sleep lasting 2-3 h during which the fowls stood in a 'tripod' position with lowered wings, or squatted; tachypnoea (180-200/min), which accompanied reserpine arousal was slowed to 80-100/minute. Despite behavioural sleep, electrocortical activity was of the alert pattern following the 0.5 and 1.0 μmol doses, but electrocortical sleep lasting about 3 h was induced by the 2.0 μmol dose. Temperature changed ±0.5°C following reserpine and dopamine. Once sleep due to dopamine waned, the fowl reverted to intense alertness. Dopamine (0.1 and 0.2 μmol) infused into the hypothalamus of 2 fowls, failed to antagonize arousal evoked by reserpine after mebanazine.

5-hydroxytryptamine In contrast to noradrenaline and dopamine, *intraventricular* 5-hydroxytryptamine hydrogen maleinate (0.25, 0.5 and 1.0 μmol) in 4 fowls did not diminish behavioural and electrocortical arousal induced by reserpine.

Young chickens Six 1-16 day chicks pretreated with mebanazine (20.0 μmol/100 g, i.p. 16 h and 2 h previously) were given reserpine (2.0 μmol/100 g, i.p.). Following initial sleep, a similar syndrome developed as described for adult fowls above, but was not obtained in 6 chicks administered reserpine only. Prenylamine, like reserpine, depletes the avian brain of noradrenaline, dopamine and 5-hydroxytryptamine (Juorio & Vogt, 1967), so this also was tested. Prenylamine (24 μmol/100 g, i.p.) evoked an arousal syndrome in 8 of 12 chicks pretreated with mebanazine (20 μmol/100 g, i.p. 16 h and 2 h previously); prenylamine alone lacked effect. Bueno *et al.* (1958) suggested that in mammals, arousal after reserpine and an amine oxidase inhibitor was related not to the absolute concentration of brain amines but to an increased 5-hydroxytryptamine : noradrenaline ratio. Experiments were repeated therefore after pretreating chicks with *p*-chlorophenylalanine, a tryptophan hydroxylase inhibitor (Koe & Weissman, 1966). Chicks (10-16 days) were given *p*-chlorophenylalanine (120 μmol/100 g, i.p. daily on 3 successive days) and mebanazine (20 μmol/100 g, i.p.) 16 h and 2 h prior to reserpine (2.0 μmol/100 g, i.p.) and observed for 4 h; a control group, pretreated as above with *p*-chlorophenylalanine, was given reserpine only. The control group squatted asleep, but in contrast those given mebanazine became extremely alert after 30-40 minutes. Arousal lasting 75-135 min, was identical to that obtained with dexamphetamine or α-methyltryptamine viz, pecking, twittering and increased initial motor activity subsiding as postural changes developed; tachypnoea (60-80/min) also developed. Once arousal abated, the chicks stood or squatted, asleep. At the end of the 4 h, chicks pretreated with mebanazine were alive, whereas 5 of the controls were dead.

Discussion

Tryptamine and α-methyltryptamine given intraventricularly or infused unilaterally into the hypothalamus of fowls evoked effects similar to their systemic administration viz, behavioural and bilateral electrocortical arousal, tachypnoea and postural changes, except that effects of these tryptamines were longer-lasting when applied centrally and effective doses were smaller. Behavioural and bilateral electrocortical arousal, but not postural changes and tachypnoea, were also induced by unilateral infusions of tryptamine or α-methyltryptamine into the mesencephalon. That tryptamine receptors, capable of mediating arousal by systemically applied agonists, were located in these areas was evident from brain transection experiments (Marley & Stephenson, 1971). 5-Hydroxytryptaminergic neurones terminate profusely in the chicken hypothalamus and mesencephalon (Ikeda & Gotoh, 1971) and the area is rich in 5-hydroxytryptamine (Juorio & Vogt, 1967); presumably tryptamine and α-methyltryptamine act postsynaptically in these areas. In favour of this idea, their effects when given into the brain were prevented by methysergide (Nisticò, unpublished observations) an antagonist of 5-hydroxytryptamine and tryptamine. Infusion of tryptamines into telencephalic cholinoceptive sites and into dopaminergic areas (paleostriatum augmentatum) were without effects.

Continuity of the brain with the spinal cord was crucial for materialization of all the above phenomena, since ipsilateral electrocortical desynchronization only was induced by infusing α-methyltryptamine unilaterally into the hypothalamus or mesencephalon of the encéphale isolé, although behavioural and bilateral electrocortical arousal were obtained following intravenous α-methyltryptamine in these preparations. An explanation for this is that in the encéphale isolè, inadequate recruitment of neurones for arousal was achieved by unilateral infusions of α-methyltryptamine in the absence of sensory input from the spinal cord, but that arousal after intravenous α-methyltryptamine accrued because of the bilateral and therefore greater recruitment of neurones. Certainly, input from the spinal cord appears more important for spontaneous arousal in fowls than in mammals, since behavioural and electrocortical sleep are uninterrupted in fowl acute encéphale isolé preparations, in the absence of drug administration (Marley & Stephenson, 1971), whereas mammalian acute preparations alternate between waking and sleeping.

5-Hydroxytryptamine given into the IIIrd ventricle or infused into the hypothalamus, induced behavioural and electrocortical sleep, although sleep was less intense than that with equimolar or even smaller doses of noradrenaline. Body temperature was elevated, although not constantly, with activation of heat-loss mechanisms once sleep abated. More significance was attached to results with the hydrogen maleinate than with other 5-hydroxytryptamine salts tested (despite intraventricular disodium maleinate provoking short-lasting behavioural arousal and elevating body temperature) because the maleinate ion, unlike the creatinine sulphate, did not influence response of cat cortical or brain-stem neurones to 5-hydroxytryptamine (Roberts & Straughan, 1967; Bradley, 1968). 5-Hydroxytryptamine had opposite behavioural and electrocortical effects to tryptamine and α-methyltryptamine. This, together with the fact that in the same fowl, tryptamine-induced arousal could be replaced by 5-hydroxytryptamine-induced sleep, and *vice versa,* implied that actions on different types of receptor was feasible. The existence of more than one type of receptor for tryptamines was proposed from studies with smooth muscle preparations (Gaddum & Picarelli, 1957), a view extended to the central nervous system (Vogt, 1968). Evidence supporting the latter came from Clineschmidt & Lotti (1974), who found certain indoleamine antagonists exhibited large differences in potency towards central effects of parenterally administered 5-hydroxytryptophan or tryptamine. However, since larger doses of tryptamine and 5-hydroxytryptamine evoked similar increases of body temperature in fowls, partial agonistic effects of the one substance on the other type of receptor has also to be countenanced.

Catecholamines infused into the hypothalamus of young (Marley & Stephenson, 1970) and adult fowls (Marley & Nisticò, 1972) induce behavioural and electrocortical sleep and lower body temperature. Noradrenaline and α-methylnoradrenaline infused into the hypothalamus, antagonized the central excitant effects of intraventricularly administered tryptamine or α-methyltryptamine, replacing them by behavioural and electrocortical sleep and even reversing the rise in body temperature so that a fall ensued. This antagonism between the two different groups could be regarded as the resultant of their opposite effects. Interactions between 5-hydroxytryptamine and noradrenaline on body temperature were not studied although it is reasonable to assume that though both induced sleep, their effects on body temperature would have been antagonistic. Opposite effects of these amines on body temperature have been noted in a number of species (see Bligh, 1973, for references), although there is no agreed interpretation of their mode of production.

Dexamphetamine given intravenously to young and adult fowls evoked behavioural and electrocortical arousal (Key & Marley, 1962) and elevated body temperature (Allen & Marley, 1967), effects identical to those of tryptamine or α-methyltryptamine and prevented or abolished by a 5-hydroxytryptamine and tryptamine antagonist, methysergide (Dewhurst & Marley, 1965a); effects of dexamphetamine on operant pecking were not prevented by methysergide but by a noradrenaline antagonist, phenoxybenzamine (Marley & Morse, 1967). Thus, dexamphetamine appears to have at least two central modes of action in fowls, a predominant one on tryptamine receptors and another mediated via noradrenaline release. Dexamphetamine given into the brain appeared also to have two different actions. Thus in adult fowls, intraventricular dexamphetamine, 0.5 μmol, elicited slight excitement and 5.0 μmol, even convulsions (Grunden & Marley, 1970), whereas infused into the hypothalamus it induced sleep, to the extent of antagonizing arousal elicited by dexamphetamine given intraperitoneally. The latter results could be ascribed to release of hypothalamic noradrenaline by an intraneuronal action of dexamphetamine, associated with impaired re-uptake of liberated noradrenaline (see Glowinski & Axelrod, 1966, for references). Compatible with this notion, noradrenaline or α-methylnoradrenaline infused into the hypothalamus, replaced the central excitant effects obtained with dexamphetamine injected intraperitoneally by behavioural and electrocortical sleep. Interestingly, even the excitant effects of tryptamine infused into the hypothalamus were preceded by brief drowsiness or sleep. After pretreating fowls with mebanazine, these excitant effects of intrahypothalamic tryptamine were attenuated or replaced by sleep (parenteral tryptamine under these circumstances evoked sustained arousal; Marley, 1968). Release by tryptamine of hypothalamic 5-hydroxytryptamine, which could cause sleep, may well account for these findings. Fuxe & Ungerstedt (1968) found in rats pretreated with a monoamine oxidase inhibitor, that tryptamine enhanced the rate of disappearance of 5-hydroxytryptamine from 5-hydroxytryptaminergic neurones and inferred that tryptamine could act presynaptically by releasing extragranular 5-hydroxytryptamine.

Behavioural, electrocortical, postural and respiratory changes resembling those obtained with dexamphetamine, tryptamine or α-methyltryptamine, given intravenously, or tryptamine and α-methyltryptamine infused into the hypothalamus, were induced in chickens given reserpine or prenylamine following pretreatment with mebanazine. The syndrome induced by reserpine was antagonized by intraventricular noradrenaline or dopamine but not by either of these substances infused into the hypothalamus, or by intraventricular 5-hydroxytryptamine. Should central noradrenaline or dopamine release by reserpine be involved in this syndrome, then our results indicate that it was unlikely to be from stores of these amines in brain areas accessible to catecholamines from the IIIrd ventricle. If, as suggested by Bueno *et al.* (1968) arousal was due to an increased ratio of brain 5-hydroxytryptamine to noradrenaline, then intraventricular catecholamines would redress this balance and even sleep ensue, as occurred in the fowls. In conflict with this thesis, the syndrome was still obtained with reserpine after doses of *p*-chlorophenylalanine which reduce brain 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in chickens to about 30% of normal (Schrold & Squires, 1971) and yet do not ostensibly interfere with catecholamine synthesis (Modigh & Svensson, 1972).

Finally, it is tempting to speculate on the relation of ion concentration and therefore ion movement at synapses, to the central effects of drugs. Behavioural arousal and elevation of body temperature similar to those evoked by tryptamine, α-methyltryptamine and dexamphetamine were elicited with intraventricular disodium maleinate. Similarly, as noted by Artunkal & Marley (unpublished observations) for fowls, the hydrochlorides of sodium or potassium, given into the brain in concentrations exceeding those normally present, induced arousal with elevation of body temperature; in contrast, centrally applied calcium (as the chloride) elicited effects simulating those of catecholamines, viz, sleep and lowering of body temperature.

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PHARMACOLOGICAL STUDIES ON SURUGATOXIN, THE TOXIC PRINCIPLE FROM JAPANESE IVORY MOLLUSC *(Babylonia japonica)*

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1 Some pharmacological properties of surugatoxin (SGTX), a purified toxic substance from the Japanese ivory mollusc (*Babylonia japonica*), have been investigated. SGTX (50 nmol/kg i.v.) produced a prolonged fall of blood pressure in anaesthetized cats. This hypotensive effect was neither blocked by atropine and propranolol nor by spinal cord transection.

2 SGTX (37-50 nmol/kg i.v.) inhibited the hypertensive and hypotensive response to 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) and to electrical stimulation of the splanchnic and vagal nerve, whereas it usually augmented the hypertensive response to adrenaline and to 4-(*m*-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium chloride (McN-A-343) in anaesthetized cats.

3 Close intra-arterial injection of SGTX (6.2-12.3 nmol/kg) to the superior cervical ganglion blocked the contractile response of the nictitating membrane to preganglionic stimulation of cervical sympathetic nerve or injected DMPP, but not to postganglionic stimulation or to injected adrenaline and McN-A-343.

4 SGTX affected neither the indirectly nor the directly stimulated response of the rat isolated phrenic nerve-diaphragm at concentrations less than 12.3 μM.

5 The effect of SGTX on the contractile response to some agonists and on the twitch response to transmural stimulation in the guinea-pig isolated ileum was investigated. At $<$12.3 μM SGTX did not depress responses to acetylcholine or histamine. The curves for nicotine- and DMPP-induced contractions were shifted to the right and depressed gradually as the concentration of SGTX was increased (12.3 nM-1.23 μM). SGTX partially inhibited the contraction induced by 5-hydroxytryptamine and the transmurally-stimulated twitch response.

6 These results suggest that SGTX has a ganglion-blocking action. The mode of anti-nicotinic action of SGTX in the guinea-pig isolated ileum seems to differ from that of hexamethonium and tetraethylammonium and to resemble more closely that of mecamylamine.

Introduction

In September 1965, food poisoning occurred from ingestion of a carnivorous gastropod, the Japanese ivory mollusc (*Babylonia japonica*), captured in the Shizuoka district of Suruga Bay. The patients complained of visual disorders, mydriasis abdominal distention, dryness of mouth, constipation and vomiting. The poisoning was apparently due to toxins, which were later demonstrated in the mid-gut gland of the mollusc (Kimura & Sugiyama, 1967).

In 1972, Kosuge, Zenda & Ochiai (1972) isolated a toxic principle in crystalline form and determined its chemical structure (molecular formula: $C_{25}H_{26}N_5O_{13}Br \cdot 7H_2O$; mol. wt: 810.53) (Figure 1). The toxin was named surugatoxin (SGTX), after Suruga Bay. SGTX causes a mydriasis with an effective minimum subcutaneous dose of 0.05 μg/g of body weight (in mice), approximately equivalent to that of atropine sulphate.

Hirayama & Gohgi (1970) have previously reported a ganglion-blocking action on the cat superior cervical ganglion of a crude extract (IS-toxin) isolated from the same species of mollusc. The action of pure SGTX has been studied further in the present experiments. We have found that SGTX produced a prolonged hypotension in anaesthetized cats and have obtained evidence that this effect might result from a ganglion-blocking action. In addition, an anti-nicotinic action of SGTX in the guinea-pig isolated ileum has been studied.

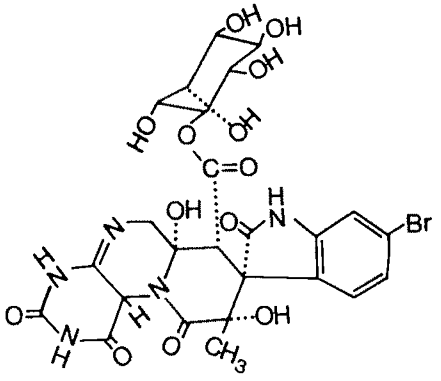

Figure 1 The chemical structure of surugatoxin.

Some accounts of this work have been given to the Japanese Pharmacological Society (Yamada & Hayashi, 1973; Yamada, Mori, Takamura & Hayashi, 1973).

Methods

Measurements of blood pressure and contractile response of nictitating membrane in cats

Cats of either sex weighing between 2.5 and 4.5 kg were anaesthetized with an intraperitoneal injection of a mixture of urethane (500 mg/kg) and chloralose (40 mg/kg). The blood pressure was recorded from the right femoral artery with a pressure transducer and the contractile response of the nictitating membrane was recorded with a force-displacement transducer connected to a multipurpose polygraph (Nihon Kohden RM-150). A force of 6 g was placed on the resting nictitating membrane in each experiment.

Drugs were administered intra-arterially close to the superior cervical ganglion or to the nictitating membrane through a polyethylene cannula inserted into the lingual artery (Trendelenburg, 1956), or intravenously into the femoral vein. Less than 0.2 ml of drug was injected through the cannula, which was flushed immediately with 0.2 ml of 0.9% w/v NaCl solution (saline). All experiments were repeated in 5-9 different animals.

Autonomic nerve stimulation

The nerves (splanchnic, vagal, or superior cervical) were placed on a pair of platinum electrodes kept moist and electrically insulated from the surrounding tissues by warm liquid paraffin. Stimulation was applied with an electrical stimulator (Nihon Kohden MSE-3R) delivering rectangular pulses of submaximal or supramaximal voltage, 1 ms duration, 5 to 30 Hz frequencies for 10 s at 2-3 min intervals. The responses recorded were: an increased and decreased blood pressure during splanchnic and vagal nerve stimulation respectively, and a contraction of the nictitating membrane during pre- and postganglionic cervical sympathetic nerve stimulation Cats sectioned at C1 of the spinal cord were artificially ventilated and the decrease of blood pressure was prevented with an intravenous injection of $BaCl_2$.

Rat phrenic nerve-diaphragm preparation

The rat phrenic nerve-diaphragm was prepared as described by Bülbring (1946). The preparation was suspended in Tyrode solution in a 30 ml organ bath at 37°C and gassed with oxygen. The responses to electrical stimulation (duration 1 ms, frequency 0.1 Hz, submaximal voltage) were isometrically recorded with a force-displacement transducer connected to a multipurpose polygraph (RM-150).

Construction of the dose-response curves for agonists in the guinea-pig isolated ileum

Male guinea-pigs weighing 300-500 g were killed by a blow on the head and a segment of the ileum was dissected at least 8 cm from the ileocaecal junction. A preparation (2-3 cm, unstretched) was suspended in Tyrode solution in a thermostatically controlled organ bath (10 ml capacity) at 37°C and gassed with oxygen. Responses of the ileum to drugs were recorded on a smoked drum with an isotonic frontal writing lever producing an approximately six-fold magnification and exerting a tension of 1 gram. The preparation was allowed to stabilize for 40 to 50 min before addition of drugs. The composition of Tyrode solution (pH: 7.6-7.8) was (g/l): NaCl, 8.0; KCl, 0.2; $CaCl_2$, 0.2; $MgCl_2$, 0.1; NaH_2PO_4, 0.05; $NaHCO_3$, 1.0; glucose, 1.0. Acetylcholine and histamine were added by means of the cumulative dose method described by van Rossum & Ariëns (1962) and van Rossum (1963) and nicotine, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) and 5-hydroxytryptamine by the single dose method. In each experiment the control dose-response curves were constructed for the agonist with five to six doses before and after the construction of the dose-response curves in the presence of SGTX or other antagonists, the second control curve being obtained 20 min after washing out the antagonists. The time of contact with agonist was 30 s and the interval between doses was 10 minutes. SGTX and some other antagonists were added to the organ bath 3-5 min before an addition of the agonist at

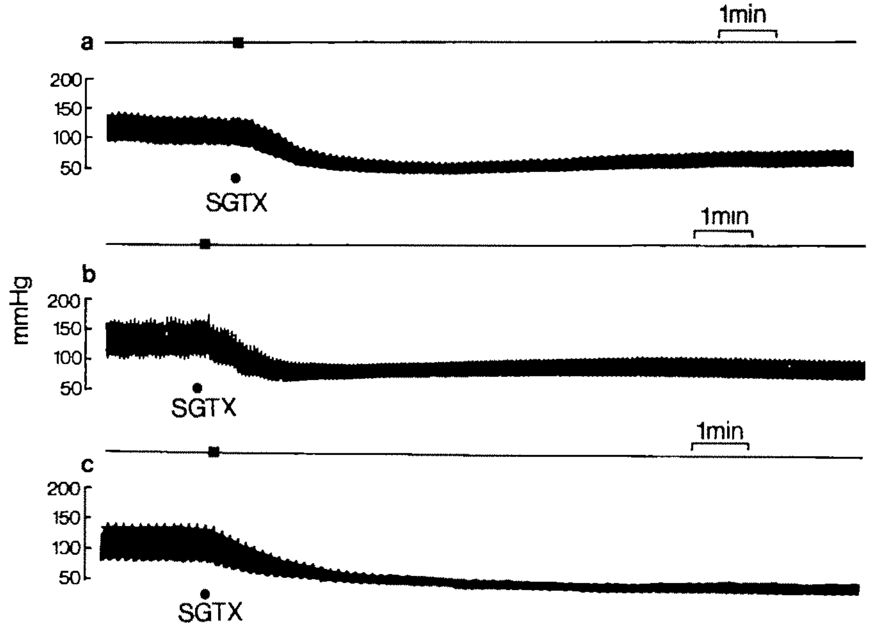

Figure 2 Absence of effect of atropine and propranolol on the hypotensive response to surugatoxin (SGTX, 50 nmol/kg i.v.) in anaesthetized cats. (a) Control, (b) after atropine (2.9 μmol/kg i.v.), (c) after propranolol (3.4 μmol/kg i.v.).

3-5 dose levels. The dose-response curves for the agonists were obtained by plotting the log concentration of agonist used against the contraction expressed as a percentage of the control maximal contraction.

Transmural stimulation

Transmural stimulation was carried out by a technique essentially similar to that described by Paton (1955, 1957). The electrodes were made of platinum and the intraluminal electrode was the anode. Rectangular pulses were used of 0.4 ms duration at a frequency of 0.1 Hz and strength sufficient to give a maximal response. The responses were recorded isometrically.

Measurement of the activity of acetylcholinesterase

The activity of the acetylcholinesterase of the guinea-pig isolated ileum was determined by the method of Guenther & Klaus (1970).

Drugs

The drugs used were obtained from the following sources: acetylcholine chloride (Daiichi), histamine diphosphate (Wako), nicotine tartrate (Wako), 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) (Aldrich), 5-hydroxytryptamine (Wako), adrenaline hydrochloride (Daiichi), 4-(*m*-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium chloride (McN-A-343) (McNeil Labs., Inc.), hexamethonium chloride (Wako), tetraethylammonium chloride (Wako), mecamylamine hydrochloride (Sigma), atropine sulphate (Merck), propranolol hydrochloride (Sumitomo), morphine hydrochloride (Takeda), cocaine hydrochloride (Sankyo), phenoxybenzamine hydrochloride (Tokyo Kasei), tetrodotoxin (Sankyo), physostigmine sulphate (Merck), urethane (Merck), α-chloralose (Wako). Crystalline SGTX was provided by Prof. Takuo Kosuge of this college. Drugs were dissolved in saline and the injected doses refer to the weights of the salts.

Results

Effects on blood pressure

Intravenous injection of SGTX (37-50 nmol/kg) produced a hypotension of 1-2 h duration in both anaesthetized and spinal cats. The hypotension was not prevented by atropine (2.9 μmol/kg i.v.) or propranolol (3.4 μmol/kg i.v.) (Figure 2).

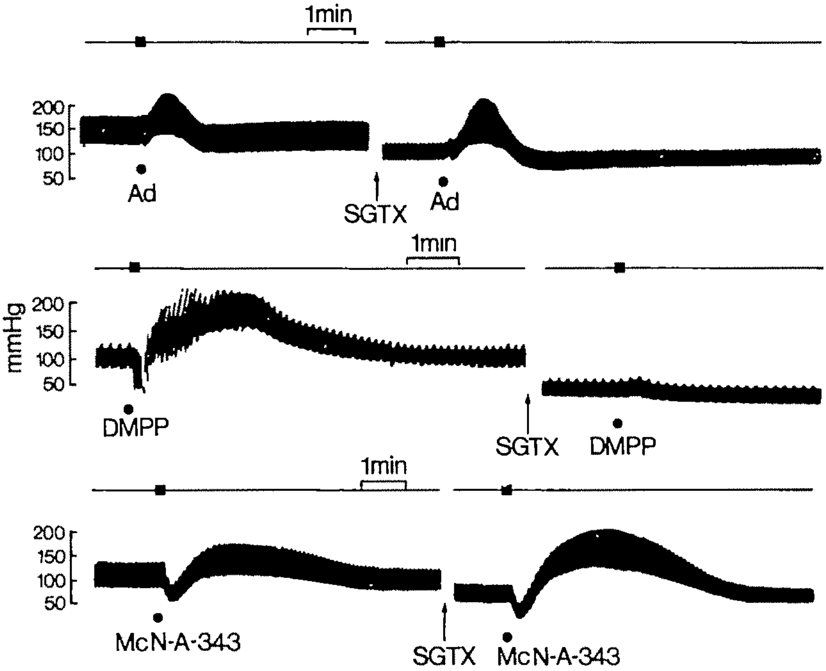

Figure 3 The effects of surugatoxin (SGTX, 50 nmol/kg i.v.) on the hypertensive response in anaesthetized cat to (a) adrenaline (Ad, 22.8 nmol/kg i.v.), (b) 1,1-dimethyl-4-phenylpiperazinium (DMPP, 94.2 nmol/kg i.v.) and (c) McN-A-343 (94.8 nmol/kg i.v.). SGTX was given 5 min before injection of agonists.

SGTX blocked the pressor response to the nicotinic ganglion-stimulant DMPP but usually enhanced the pressor response to adrenaline and the secondary pressor response to the muscarinic ganglion-stimulant McN-A-343 (Figure 3).

SGTX reduced the pressor or depressor response to both splanchnic and vagal nerve stimulation (Figure 4).

Effects on ganglionic transmission

Intravenously-injected SGTX (50 nmol/kg) inhibited the contraction of the nictitating membrane produced by preganglionic stimulation of the cervical sympathetic nerve without affecting the response to postganglionic stimulation of the nerve or to intravenously injected adrenaline (Figure 5). Close-arterial injection of SGTX (6.2-12.3 nmol/kg) to the superior cervical ganglion blocked the response of the nictitating membrane to preganglionic stimulation and to close-arterial injection of DMPP (6.3 nmol/kg), but not that to McN-A-343 (31.6 nmol/kg). Close-arterial injection of SGTX (12.3 nmol/kg) did not affect the response to postganglionic stimulation or adrenaline (injected into the external carotid artery). The effect of SGTX on ganglionic transmission was compared quantitatively with that of hexamethonium and mecamylamine in nine experiments (Figure 6). The molar potency of SGTX was 20 to 30 times greater than that of mecamylamine and approximately 50 times greater than that of hexamethonium.

Neuromuscular transmission

SGTX did not reduce the amplitude of the contractions of the rat isolated diaphragm preparation to the direct (muscle) or indirect (phrenic nerve) stimulation at a concentration of 12.3 μM.

Effects on the guinea-pig isolated ileum

SGTX itself had very little effect on the tone of the ileum at concentrations less than 12.3 μM.

The dose-response curves for acetylcholine and histamine were not affected by SGTX at concentrations below 12.3 μM. Bell-shaped dose-response curves were obtained for nicotine (2.0 μM-2.0 mM) and DMPP (0.94 μM-0.94 mM) as previously described by Trendelenburg (1961)

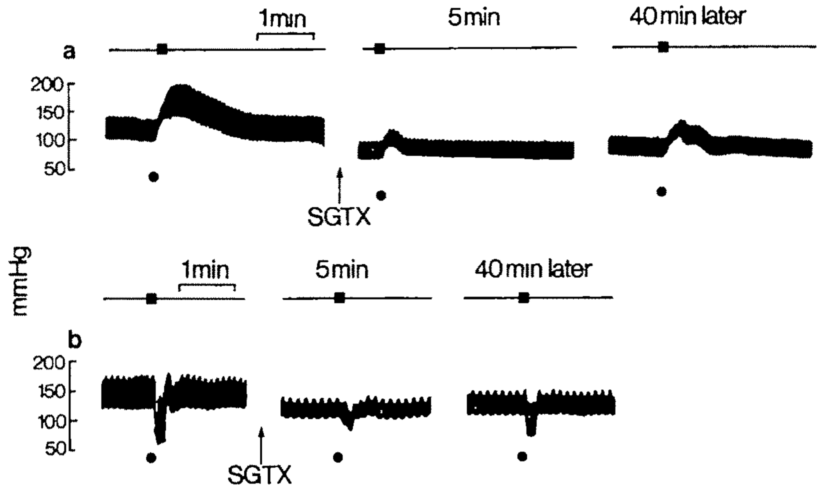

Figure 4 The effects of surugatoxin (SGTX, 50 nmol/kg i.v.) on (a) the hypertensive and (b) hypotensive responses to electrical stimulation of splanchnic and vagal nerves respectively in anaesthetized cats. Dots: supramaximal stimulation for 10 seconds. (a) The response to splanchnic nerve stimulation, (b) the response to vagal nerve stimulation.

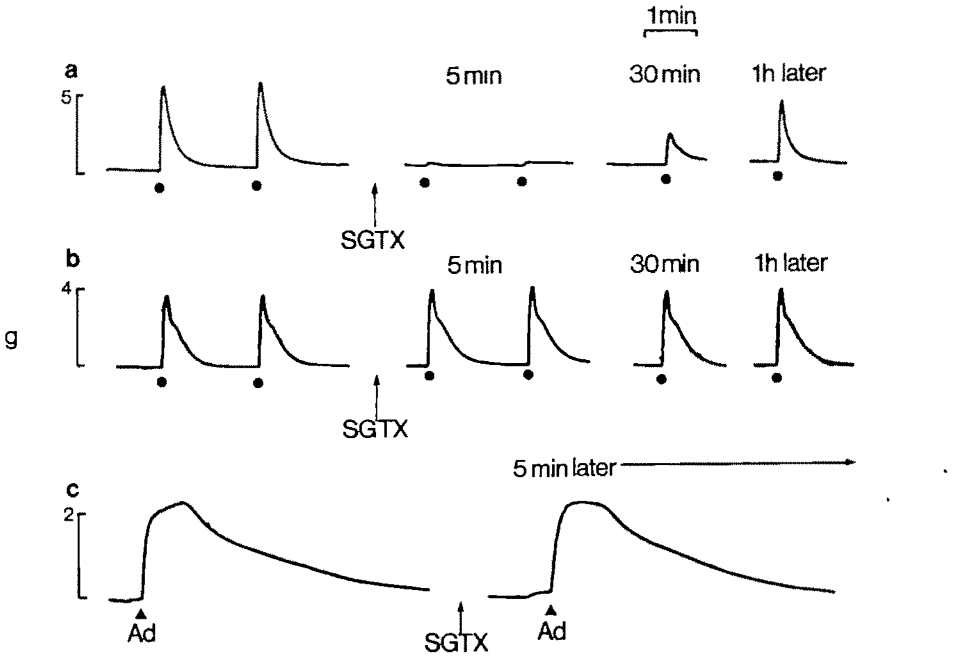

Figure 5 The effects of surugatoxin (SGTX, 50 nmol/kg i.v.) on the contractile responses of the nictitating membrane to (a) preganglionic and (b) postganglionic stimulation of the cervical sympathethic nerve and to (c) injected adrenaline (Ad, 45.5 nmol/kg i.v.). Dots: (a) supramaximal preganglionic and (b) submaximal postganglionic stimulation (30 and 15 Hz) for 10 s at 2 min intervals.

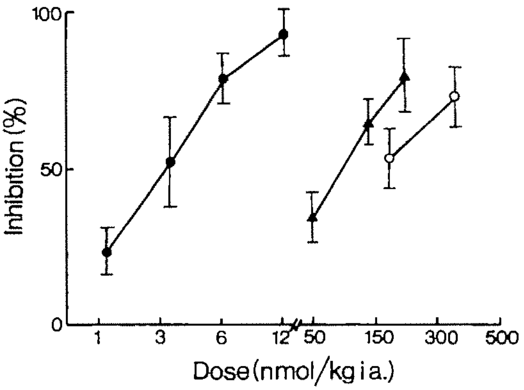

Figure 6 The effects of close-arterial surugatoxin (●), mecamylamine (▲) and hexamethonium (○) on the contractile response of the nictitating membrane to supramaximal preganglionic stimulation of the cervical sympathetic nerve. Ordinates: average % of inhibition (n = 6-9). Abscissae: doses of surugatoxin, hexamethonium and mecamylamine (injected close-arterially to the superior cervical ganglion). Vertical bars indicate s.e mean.

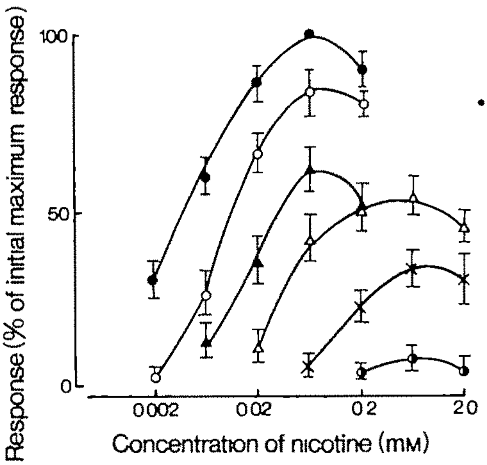

Figure 7 The effects of surugatoxin (SGTX) on the dose-response curves for nicotine in the guinea-pig isolated ileum. (●) Control, in the presence of SGTX, (○) 12.3 nM, (▲) 36.9 nM, (△) 123 nM, (x) 369 nM, (○) 1.23 μM Each point represents the mean of at least six experiments and vertical bars indicate s.e. mean.

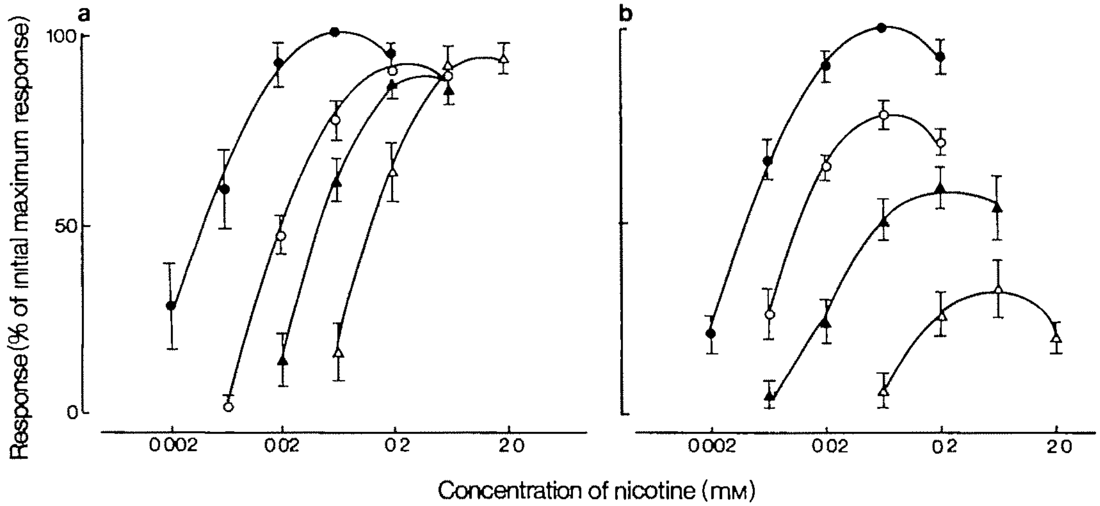

Figure 8 The effects of (a) hexamethonium and (b) mecamylamine on the dose-response curves for nicotine in the guinea-pig isolated ileum. (●) Control; in the presence of (a) hexamethonium (○) 3.66 μM, (▲) 10.98 μM, (△) 36.6 μM and (b) mecamylamine (○) 0.49 μM, (▲) 1.47 μM, (△) 4.9 μM. Each point represents the mean of at least five experiments and vertical bars indicate s.e mean.

and van Rossum (1962). The dose-response curves for nicotine and DMPP were shifted to the right and depressed gradually as the concentration of SGTX was increased from 12.3 nM to 1.23 μM (Figure 7). The contraction produced by either nicotine or DMPP was reduced to less than 10% of the maximal contraction with a concentration of SGTX of 1.23 μM.

The anti-nicotinic action of SGTX on the guinea-pig isolated ileum was compared with that of hexamethonium, tetraethylammonium and mecamylamine. Hexamethonium (3.66-36.6 μM) and tetraethylammonium (6.0-60.0 μM) shifted the curve for nicotine to the right without depression (Figure 8a), whereas mecamylamine (0.49-4.9 μM) caused a shift to the right with

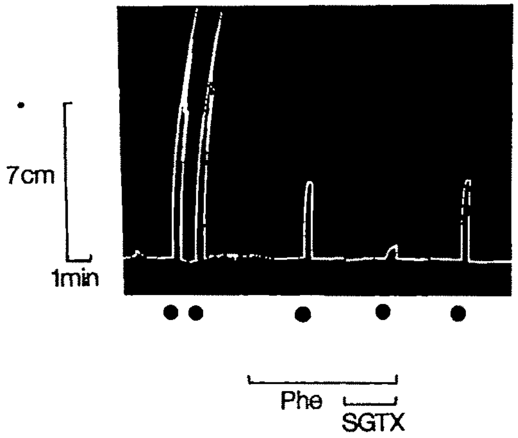

Figure 9 The effect of phenoxybenzamine (Phe) and surugatoxin (SGTX) on the contraction induced by 5-hydroxytryptamine in the guinea-pig isolated ileum. Dots indicate an addition of 5-hydroxytryptamine (1.7 μM) and the bars indicate the presence of phenoxybenzamine (0.31 μM for 30 min) and SGTX (1.23 μM). Time marker: 1 min, vertical calibration: 7 cm on kymograph tracing.

gradual depression resembling SGTX (Figure 8b). These results obtained with hexamethonium and mecamylamine are consistent with those in the guinea-pig isolated jejunum reported by Ariëns (1964). In order to quantify the results pA_2 and pD'_2 values for these ganglion-blocking agents were calculated as described by previous workers (Schild, 1949; Ariëns & van Rossum, 1957). pA_2 values for hexamethonium, tetraethylammonium, mecamylamine and SGTX against nicotine were 5.95 ± 0.04 ($n = 4$), 5.34 ± 0.09 ($n = 3$), 6.68 ($n = 2$) and 8.10 ($n = 2$) respectively. pD'_2 values for mecamylamine and SGTX were 5.70 ± 0.05 ($n = 4$) and 7.00 ± 0.11 ($n = 5$) (mean ± s.e. mean). The pA_2 value for hexamethonium in the present experiment was reasonably close to that (5.8) obtained by van Rossum (1962). These data indicate that the anti-nicotinic action of SGTX in the guinea-pig isolated ileum is approximately 100 times greater than that of hexamethonium or tetraethylammonium, and 20 to 30 times greater than that of mecamylamine.

SGTX (0.12-1.24 μM) partially (40-50%) inhibited the contraction due to 5-hydroxytryptamine. An attempt was made to find out which of the 5-hydroxytryptamine receptors (D- or M-receptors, Gaddum & Picarelli, 1957) was inhibited by SGTX. As shown in Figure 9, the contraction of the ileum to 5-hydroxytryptamine was almost completely inhibited on the addition of SGTX in the presence of phenoxybenzamine. After washing the preparation, only the inhibition due to SGTX disappeared and that due to phenoxybenzamine still remained. In contrast, after morphine-inhibition, SGTX (1.23 μM) produced little or no further depression of the response to 5-hydroxytryptamine.

Contraction of the ileum induced by transmural

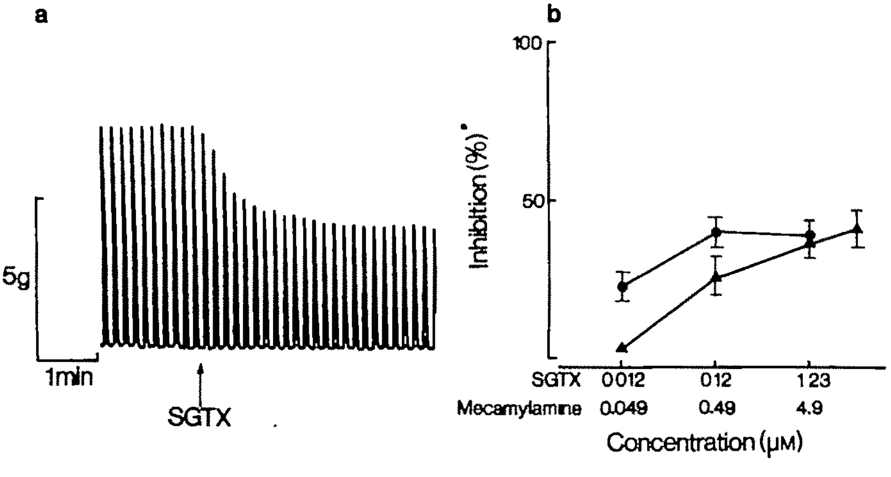

Figure 10 The effect of surugatoxin (SGTX) and mecamylamine on the twitch response to transmural stimulation in the guinea-pig isolated ileum. (a) The inhibitory action of SGTX (123 nM) on the twitch response. Time marker: 1 min, vertical calibration: 5 g. (b) The dose-inhibition curves for SGTX (●) and mecamylamine (▲) on the twitch response. Each point represents the mean of at least five experiments and vertical bars indicate s.e. mean.

electrical stimulation was blocked by morphine (8.0 μM), adenosine (0.15 μM), tetrodotoxin (0.31 μM) and atropine (0.14 μM) and potentiated by physostigmine (0.15 μM). Hexamethonium and tetraethylammonium had very little effect on the response at concentrations less than 36.6 μM These results accord with previous findings (Paton, 1955, 1957; Scriabine & Peklak, 1970) that contractions of the guinea-pig isolated ileum in response to transmural stimulation involved the excitation of postganglionic cholinergic nerves and the consequent release of acetylcholine. SGTX partially inhibited, by approximately 20-40%, the transmurally induced contractions of the ileum at concentrations of 12.3 nM-1.23 μM. Mecamylamine (0.49-14.7 μM) exerted a similar effect (Figure 10).

SGTX and mecamylamine had no effect on the acetylcholinesterase activity of the ileum at concentrations less than 1.23 μM and 49.0 μM respectively.

Discussion

The experiments with anaesthetized cats indicate that SGTX exerts a ganglion-blocking action. The mechanism of block appears to be similar to that of hexamethonium in that (a) only nicotinic receptors are blocked and (b) blockade is not preceded by ganglionic stimulation. We have not established whether SGTX exerts any prejunctional effect, but this seems unlikely because neuromuscular transmission was not impaired.

In the guinea-pig ileum, SGTX appears to act preferentially upon the intrinsic nerve supply of the ileum rather than on the smooth muscle, since it antagonized the effects of nicotine and DMPP without affecting direct muscle responses to acetylcholine and histamine. Antagonism to nicotine and DMPP may largely result from ganglion-blockade. However, this antagonism did not appear to be strictly competitive in that the maximum responses to nicotine and DMPP were depressed, perhaps resulting from an additional component of activity upon the postganglionic nerves *per se* rather than the nicotinic receptors alone. This is further indicated by (a) partial depression of the response to transmural stimulation and (b) inhibition of the phenoxybenzamine-resistant component of the response to 5-hydroxytryptamine, which has been attributed to stimulation of intramural nerves (Gaddum & Picarelli, 1957). In these respects SGTX differs from hexamethonium and more closely resembles mecamylamine.

Most of the clinical symptoms resulting from ingestion of the mollusc appear to be mediated by ganglion-blockade at various sites; visual disorder and mydriasis, due to ciliary ganglion blockade, dryness of mouth, due to submaxillary and otic ganglion blockade, and constipation and abdominal distention due to blockade of the intrinsic nerve in the intestine.

A ganglion-blocking action of a crude extract from this species has been reported previously by Hirayama & Gohgi (1970). However, the active principle which they used (IS-toxin) appears to be different from SGTX in terms of elemental composition and molecular weight. SGTX has the formula $C_{25}H_{26}N_5O_{13}Br \cdot 7H_2O$ (mol. wt 810.53), whereas the IS-toxin has a formula $C_{22}H_{36}N_4O_{14}Br$. (mol. wt 660) and the chemical structure is unknown. Further, the two toxins are eluted in different fractions from a CM-Sephadex C-25 column. Consequently, there appear to be two separate toxins in this species with similar pharmacological properties.

Structurally, SGTX differs appreciably from other known ganglion-blocking agents. Its potency is 50 to 100 times greater than that of hexamethonium. In these respects, SGTX appears to be of some pharmacological interest as a new type of ganglion-blocking agent.

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INVESTIGATION OF THE VASODILATOR AND ANTISECRETORY ROLE OF PROSTAGLANDINS IN THE RAT GASTRIC MUCOSA BY USE OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

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1 The effects of non-steroidal anti-inflammatory drugs on gastric acid secretion and mucosal blood flow were studied in the rat.

2 Indomethacin, in ulcerogenic doses, caused a dose-dependent rise in pentagastrin-stimulated acid secretion, but decreased mucosal blood flow per unit acid secretion.

3 . During resting conditions, indomethacin had no significant effect on acid output, but reduced mucosal blood flow.

4 Pretreatment with indomethacin, phenylbutazone or meclofenamate potentiated the secretory response to dibutyryl cyclic adenosine 3′,5′-monophosphate.

5 Indomethacin markedly reduced the mucosal prostaglandin-like activity at a time when mucosal erosion formation had reached steady levels.

6 These results provide evidence that prostaglandins have a local role in the regulation of blood flow and acid secretion in the rat gastric mucosa, and suggest that non-steroidal anti-inflammatory drugs cause mucosal erosions by disrupting these processes.

Introduction

Prostaglandins of the E and A series, which inhibit gastric acid secretion, have a direct vasodilator action on the rat gastric mucosa (Main & Whittle, 1973a). These findings, together with the increased release of mucosal prostaglandins during secretory stimulation (Shaw & Ramwell, 1968) raised the possibility that prostaglandins have a physiological role, not only as negative feed-back inhibitors of acid secretion, but also as mediators of functional vasodilatation in the gastric mucosa.

In the present study, these hypotheses have been tested by the use of indomethacin and other non-steroidal anti-inflammatory drugs which inhibit prostaglandin synthesis (Vane, 1971). We have also investigated the relationship between inhibition of gastric mucosal prostaglandin synthesis and the formation of mucosal erosions by these drugs.

Preliminary accounts of this work have been presented to the British Pharmacological Society (Main & Whittle, 1973b, c).

Methods

Measurement of gastric acid secretion and mucosal blood flow

Acid output into the perfused gastric lumen of the urethane-anaesthetized rat was measured, and mucosal blood flow was determined simultaneously by the [^{14}C]-aniline clearance technique (Main & Whittle, 1973d).

Assessment of mucosal erosions

Female Wistar rats (200-250 g), starved for 18 h but allowed water, were killed at various time intervals following drug treatment and the stomachs removed. After being opened along the lesser curvature, the stomachs were rinsed under a stream of water and pinned flat on a cork-board. The stomachs were coded to prevent observer bias and studied with the aid of a binocular microscope (x10). Erosions, which formed only in the glandular mucosa, were counted and each one given a severity rating on a 1 to 3 scale (1 = less than 1 mm; 2 = 1 to 2 mm; 3 = greater than 2 mm). The overall total, divided by a factor of 10, was designated the 'ulcer index' for that stomach.

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Extraction and estimation of prostaglandin-like material from rat gastric perfusate and mucosae

Gastric perfusate was collected directly into glass cylinders stored on ice. An aliquot (0.5 ml) was taken for estimation of the acid content, whilst the remainder (acidified to pH 4 with citric acid if necessary) was extracted twice with ethyl acetate and the pooled organic phase evaporated to dryness under reduced pressure at 45° C. The residue was either immediately bioassayed or stored at –15° C until required.

Gastric mucosae, from rapidly dissected stomachs washed in ice-cold saline (0.9% w/v NaCl solution), were scraped from the underlying muscle layers with a chilled glass slide and placed immediately in 96% aqueous ethanol at 0° C containing 100 nCi [^{3}H]-prostaglandin E_1 (0.4 ng). This procedure lasted about two minutes. The tissue was rapidly homogenized, extracted into ethyl acetate, pH 8 phosphate buffer and 67% aqueous ethanol as described by Horton & Main (1967), and the biological activity determined on the rat isolated stomach strip. The efficiency of extraction, as estimated by the radioactivity in the final residue, was 68 ± 2% ($n = 7$). Thin-layer chromatography of mucosal extracts was carried out on aluminium-backed plates (0.2 mm layer of silica gel F_{254}, fast-running; Merck). Silver nitrate impregnated plates were prepared by immersing in an alcoholic solution of silver nitrate B.P. (approximately 4% in ethanol) for 1 h and drying at room temperature. The AI and AII solvent systems of Gréen & Samuelsson (1964) were used.

Measurement of binding of indomethacin and dibutyryl cyclic adenosine 3′,5′-monophosphate (dbcAMP) to plasma protein

Five ml of bovine serum albumin (4% in pH 7.4 sodium phosphate buffer), containing indomethacin (50-300 µg/ml), dbcAMP (50 µg/ml) or a mixture of both, was pipetted into Visking tubing and dialysed against 10 ml pH 7.4 buffer maintained at 37° C in a shaking water bath. After 24 h, the concentration of indomethacin (absorbance measured at 265 nm) and dbcAMP (measured at 273 nm) in the dialysate was determined with a u.v.-spectrophotometer (Unicam SP.800 B).

Statistical analysis

Results are shown as the mean ± standard error of the mean, where (n) is the number of values in the group. The significance of the data was evaluated using Student's t test for unpaired or paired data where appropriate. $P < 0.05$ was taken as significant.

Drugs

Pentagastrin (Peptavlon, I.C.I.); N^6-2′-*O*-dibutyryl cyclic adenosine 3′,5′-monophosphate (Boehringer); prostaglandin E_1-[5,6-^{3}H] (New England Nuclear). Indomethacin (Merck, Sharp & Dohme, Ltd) was added to saline containing a stoichiometrically-equivalent quantity of sodium bicarbonate, and dissolved with the aid of the dropwise addition of 0.1 N NaOH (taking care not to exceed pH 8) to give a final concentration of 4-5 mg/ml. Before use, the concentration and spectrum of the solution was determined by spectrophotometry (between 200 and 400 nm). Phenylbutazone (Geigy Pharmaceuticals) was suspended in carboxy-methyl cellulose (1% in distilled water), to give a final concentration of 100 mg/ml. In control experiments, the appropriate vehicle was administered. Sodium meclofenamate (Parke-Davis & Co.) was dissolved in saline. Prostaglandins were supplied by the Upjohn Co., Kalamazoo, U.S.A.

Results

Effects of indomethacin during basal and pentagastrin-stimulated acid secretion

During resting conditions, indomethacin (30 mg/kg) injected intravenously over a period of 5-10 min, did not significantly alter acid output ($P > 0.1$) but caused a gradual fall in mucosal blood flow (Figure 1). Mucosal blood flow had fallen significantly ($P < 0.02$) by a mean of 49.2 ± 9.3% ($n = 4$) 2 h after administration as shown in Figure 2.

Indomethacin (20-40 mg/kg i.v.) had no significant effect on systemic arterial blood pressure (eight observations). During the gastric perfusion of acidic saline (0.01 N HCl) under basal conditions, indomethacin (30 mg/kg i.v.) did not increase the back-diffusion of hydrogen-ions (two observations).

During the steady submaximal acid secretory response to an infusion of pentagastrin (0.20-0.33 µg kg^{-1} min^{-1}), indomethacin (20 and 40 mg/kg i.v.) caused a gradual increase in acid output during the following 2 to 3 hours. The acid output measured after 2 h had increased significantly from the control values ($P < 0.02$ for both series) in a dose-dependent manner (Figure 2). Although an increase in mucosal blood flow accompanied the rise in acid, the ratio of mucosal blood flow to acid output was decreased

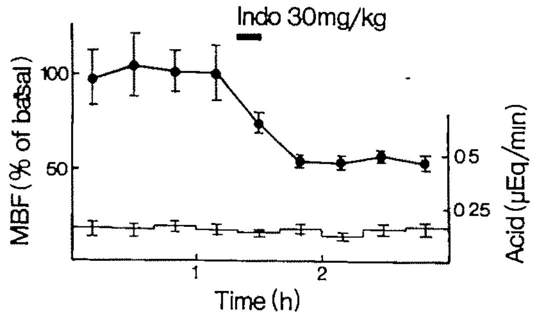

Figure 1 Effects of intravenously-administered indomethacin (Indo) on resting gastric mucosal blood flow (MBF) (●) and acid secretion (⌐¬) in the rat. The results are the mean of four experiments. Vertical bars show s.e. mean.

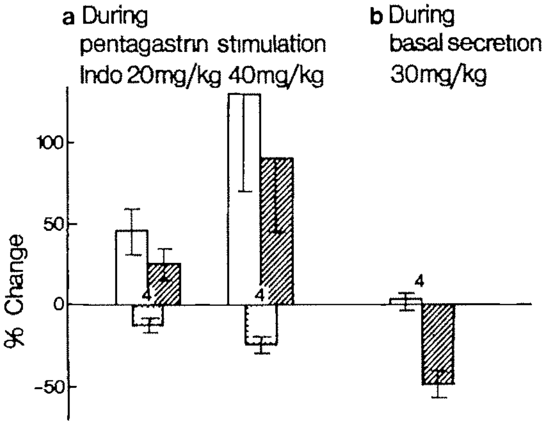

Figure 2 Effects of intravenously-administered indomethacin on gastric acid secretion (open columns), mucosal blood flow (hatched columns) and mucosal blood flow per unit acid secretion (dotted columns) during (a) pentagastrin stimulation, and (b) basal secretion. Results, expressed as % change from control value, are given as the mean of four experiments in each series. Vertical bars show s.e. mean.

($P < 0.05$), as shown in Figure 2. These effects were usually followed by the appearance of blood in the gastric perfusate and mucosal erosions were observed post mortem.

Effects of indomethacin on dbcAMP-induced acid secretion

Intravenous injection of dbcAMP during basal conditions caused a small but reproducible increase in acid output lasting approximately 80 min (Main & Whittle, 1974a). After control responses to dbcAMP (20 mg/kg i.v.) had been obtained, indomethacin (10-30 mg/kg) was injected intravenously. Injection of dbcAMP 1 h later caused a marked increase in acid output. The results, expressed as the increase in acid output (μEq) from basal levels during the 80 min following injection of dbcAMP, are shown in Figure 3. The dbcAMP-induced secretory responses following indomethacin administration (10-30 mg/kg) were significantly greater than the control responses ($P < 0.001$ for each group). The response following a 30 mg/kg dose of indomethacin was significantly greater than that with 10 mg/kg ($P < 0.001$).

Pretreatment of rats with indomethacin (15 mg/kg) injected subcutaneously 6 or 24 h before stimulation (that is, 4 or 22 h before surgery), in a dose which produced gastric mucosal erosions, significantly ($P < 0.001$) potentiated the secretory response to dbcAMP (Figure 3b).

Theophylline (20 mg/kg i.v., 1 h prior to dbcAMP-stimulation) significantly ($P < 0.001$) augmented the potentiating effect of indomethacin (Figure 3b) and gastric bleeding occurred in each experiment during these very high secretory rates. This dose of theophylline, which had no consistent effect on basal or pentagastrin-stimulated secretion (Main & Whittle, 1974a) did not significantly alter the response to dbcAMP alone, in three experiments. In another experiment, where theophylline increased basal secretion three-fold, the dbcAMP response was increased four-fold.

Effects of sodium meclofenamate and phenylbutazone

Intravenous administration of sodium meclofenamate (30 mg/kg) caused a significant ($P < 0.001$) increase of the secretory response to dbcAMP (20 mg/kg) injected 1 h later (Figure 3a).

Pretreatment of rats with phenylbutazone (100 mg/kg s.c.) 6 h prior to stimulation, significantly ($P < 0.001$) increased the dbcAMP-induced secretory response (Figure 3b). This dose of phenylbutazone was found to produce gastric mucosal erosions within 6 h of subcutaneous administration.

Production of gastric erosions by indomethacin

Indomethacin (5-30 mg/kg) was administered subcutaneously to rats (eight per group), and after a predetermined time interval the ulcer index of each rat stomach was assessed. Indomethacin in a dose of 15 mg/kg produced a submaximal degree

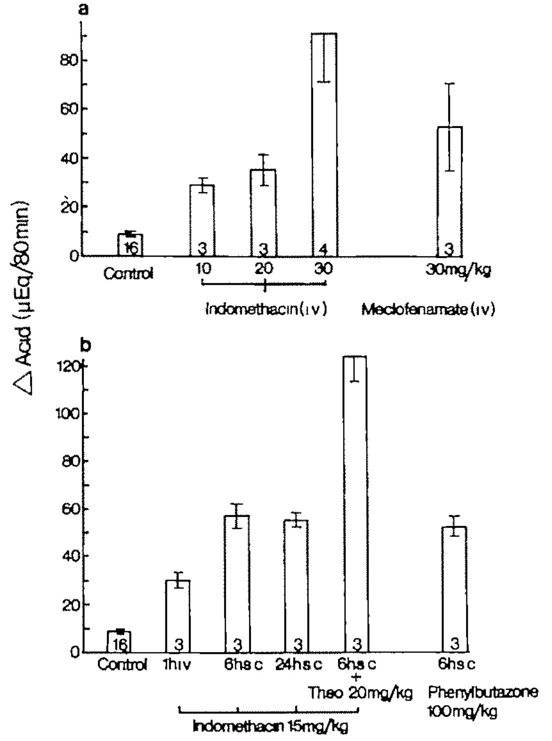

Figure 3 Effects of anti-inflammatory drugs on the secretory response to intravenous dibutyryl cyclic adenosine 3′,5′-monophosphate (dbcAMP) (20 mg/kg) during basal secretion in the rat Acid output (μEq) is the increase from basal during the 80 min following dbcAMP. All results are expressed as mean with s.e. (vertical bars) where n is the number of experiments.

of gastric ulceration after 6 h, which remained almost constant during the following 12 to 24 h (Figure 4).

Prostaglandin-like activity in the rat gastric perfusate and mucosa

No prostaglandin-like activity (less than 0.06 ng/min in terms of prostaglandin E_2) could be detected in extracts of gastric perfusate collected over periods of 20 to 120 min during either basal, pentagastrin- or histamine-stimulated acid secretion (14 determinations from six experiments). This lack of activity was not due to inadequate extraction, since $[^3H]$-prostaglandin E_1 or E_2 added to the acidic perfusate (to give a final concentration of 1 to 10 ng/ml), was completely recovered (99 ± 2%, $n = 9$). Further, no loss of biological activity was observed when prostaglandin E_2 (10 ng/ml) was incubated in gastric perfusate (pH 2) for 40 min at room temperature (three experiments).

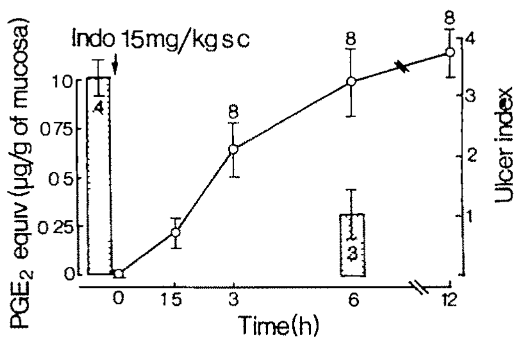

Figure 4 Effects of indomethacin on prostaglandin-like activity (dotted columns) and erosion formation (o) in the rat gastric mucosa. Results are expressed as the mean with s.e. (vertical bars) where n is the number of values in the group.

In three experiments, where $[^3H]$-prostaglandin E_1 (4nCi/ml, 10 ng/ml; 0.2 ml/min) was perfused through the gastric lumen for up to 200 min during basal or stimulated secretion, 86 ± 5% ($n = 14$) of the biological activity, and 87 ± 1% ($n = 23$) of the radioactivity was recovered in the samples of perfusate. In a further two experiments during pentagastrin-stimulated acid secretion, $[^3H]$-prostaglandin E_1 ($2\ \mu g\ kg^{-1}\ min^{-1}$, $0.2\ \mu Ci\ kg^{-1}\ min^{-1}$) was infused intravenously for 40 minutes The inhibition of acid output was not accompanied by an output of biologically active prostaglandin-like material into the perfusate (<0.1 ng/min), and less than 1% of the administered radioactivity appeared in the perfusate during the following 2 hours.

Gastric mucosal tissue from three rats was used in each determination of prostaglandin-like activity. After chromatographic separation on the AI system, 85% of the biological activity was located in the prostaglandin E zone. When chromatographed on the AII system, 70% of this biological activity was located in the prostaglandin E_2 zone. The prostaglandin-like activity of the mucosa, expressed in terms of prostaglandin E_2-equivalents (after correction for efficiency of extraction) was 1.03 ± 0.09 μg/g of mucosa ($n = 4$). This was significantly ($P < 0.01$) reduced to 0.32 ± 0.13 μg/g of mucosa, ($n = 3$) 6 h following indomethacin (15 mg/kg s.c.), as shown in Figure 4.

Binding of indomethacin and dbcAMP to plasma protein

Indomethacin in concentrations of 50 to 300 μg/ml was 96 ± 1% ($n = 6$) bound to bovine serum albumin as determined by equilibrium dialysis, whereas dbcAMP (50 μg/ml) was not bound (less than 3% bound, $n = 4$).

Discussion

Parenteral administration of indomethacin, which inhibits prostaglandin synthesis (Vane, 1971), caused a fall in rat gastric mucosal blood flow during resting conditions and a reduction in mucosal blood flow per unit acid secretion during pentagastrin stimulation. This supports our concept that prostaglandins have a physiological role as vasodilators in the gastric mucosa of this species.

However, the mucosa still retained much of its ability to vasodilate during stimulation of acid secretion. This could be due to incomplete inhibition of prostaglandin synthesis in the mucosa. Alternatively, other vasodilators such as histamine, adenosine metabolites or CO_2 play an important role or may be capable of doing so under conditions of reduced prostaglandin synthesis, although the mucosal blood flow which they maintain may be inadequate. Comparable studies in other species have not yet been reported. In the dog, a decrease in resting mucosal blood flow following intragastric administration of aspirin was observed (O'Brien & Silen, 1973) although in an earlier study, no such effect was found (Augur, 1970).

Indomethacin, in ulcerogenic doses, had no significant effect on basal acid output. This agrees with the results of previous studies in the pylorus-ligated rat (Catanese, Lisciani & Silvestrini, 1970; Lee, Mollison & Cheng, 1971), though pylorus ligation may not be equatable with basal conditions in the gastric perfusion preparation which yields more precise and meaningful information on secretory processes. Indomethacin significantly increased pentagastrin-stimulated secretion, which is compatible with a negative feed-back inhibitory role of prostaglandins in the mucosa, operative during stimulated secretion. An increase in basal and pentagastrin-stimulated secretion has been observed in pylorus-ligated rats, following chronic administration of 5, 8, 11, 14-eicosa tetraynoic acid (Shaw, Jessup & Ramwell, 1972) which inhibits prostaglandin synthesis. In contrast, indomethacin failed to augment pentagastrin- or histamine-stimulated acid secretion in man (Winship & Bernard, 1970; Bennett, Stamford & Unger, 1973) and decreased secretion in the dog (Nicoloff, 1968). However, before these results can be attributed to species differences in the effects of indomethacin and in the role of mucosal prostaglandins, other factors such as the dose and route of administration must be considered. Although back diffusion of hydrogen-ions did not occur with the gastric perfusion technique used in our experiments, this phenomenon has often been observed following administration of aspirin-like drugs (Chvasta & Cooke, 1972), and may have masked significant changes in acid secretion in dog and man.

Since cyclic adenosine 3′,5′-monophosphate (cyclic AMP) may be the intracellular mediator of gastric acid secretion, it was considered of value to investigate the effects of indomethacin on the secretory response induced by the dibutyryl analogue of cyclic AMP (dbcAMP). When injected during basal secretion, dbcAMP gives a small but reproducible acid secretory response and hence any changes can be readily detected. Indomethacin or the other potent non-steroidal anti-inflammatory drugs, sodium meclofenamate or phenylbutazone, caused a marked potentiation of the dbcAMP-induced responses. These effects were not caused by simple displacement of dbcAMP by these highly plasma protein-bound drugs.

Prostaglandins may depress the formation of cyclic AMP in the gastric mucosa by an action on the adenyl cyclase system (Way & Durbin, 1969; Main & Whittle, 1974a). Thus, a reduction in mucosal prostaglandin activity by aspirin-like drugs would allow the endogenous level or turnover of cyclic AMP to increase and hence augment the acid secretory response This is supported by recent findings that aspirin stimulates rat mucosal adenyl cyclase activity (Mangla, Kim & Rubulis, 1974). Whether the potentiation of the dbcAMP-induced acid secretion can likewise be attributed to such an action, awaits further investigation into the nature of the secretory response to dbcAMP (Main & Whittle, 1974a). The mechanism by which theophylline, a phosphodiesterase inhibitor, further augments the indomethacin-potentiated secretory response to dbcAMP is not clear, since dbcAMP is resistant to this enzyme (Cehovic, Posternak & Charollais, 1972).

The observed effects of the non-steroidal anti-inflammatory drugs may be attributable to properties other than their ability to inhibit prostaglandin synthesis. Indomethacin and several similar drugs inhibit phosphodiesterases *in vitro* (Flores & Sharp, 1972; Karppanen & Puurunen, 1974; Newcombe, Thanassi & Ciosek, 1974), though the concentrations required are much higher than those which inhibit prostaglandin synthesis, and may be of limited significance *in vivo*. Furthermore, the potent phosphodiesterase inhibitor, theophylline, had no consistent effect on the secretory response to either pentagastrin or dbcAMP in the rat. The fall in mucosal blood flow is unlikely to result from increased cyclic AMP levels since cyclic AMP causes vasodilatation of the mucosa (Main & Whittle, 1974a). It has been suggested that the mucosal damage which follows gastric instillation of salicylic acid in the pylorus-ligated rat is due to histamine release from the mucosa (Johnson, 1966). This release could not explain the reduced mucosal blood flow observed in the present experiments, and we have found that parenterally-administered indomethacin had no significant effect on the histamine output into the rat gastric lumen during basal or pentagastrin-stimulated acid secretion (Main & Whittle, unpublished results).

If non-steroidal anti-inflammatory drugs are acting by reducing the formation of mucosal prostaglandins, then the output of prostaglandin-like material into the rat gastric perfusate, observed by Shaw & Ramwell (1968), might be expected to decrease prior to the changes in acid secretion and mucosal blood flow. However, this has remained untested since, in the current investigation, no prostaglandin-like activity could be detected in gastric perfusate extracts. Likewise, very little prostaglandin-like material could be detected in human gastric juice (Horton, Main, Thompson & Wright, 1968; Bennett *et al.*, 1973). This may reflect low permeability of the gastric mucosa to prostaglandins, since, following intravenous injection of [^{3}H]-prostaglandin E_1, very little radioactivity could be detected in the gastric perfusate. Inactivation or loss of prostaglandins following their release from the mucosa into the lumen is unlikely to account for our results, since when low concentrations of [^{3}H]-prostaglandin E_1 or E_2 were perfused through the gastric lumen, approximately 90% of the biological activity and radioactivity were recovered from the perfusate. In contrast, Shaw & Ramwell (1968), using a similar technique, detected prostaglandin-like activity in the gastric perfusate despite a 75% loss of perfused prostaglandins. We cannot exclude the possibility that A-prostaglandins or metabolites of E-prostaglandins which are undetectable by biological assay, were present in the gastric perfusate in our experiments. Both A and E prostaglandins may be present in dog gastric secretion (Dozois & Thompson, 1974) though the amounts are very low and, as with prostaglandin E_2 in man (Bennett *et al.*, 1973), no significant change in the concentration and output has been detected during stimulation of acid secretion. Nevertheless, lack of prostaglandin release into the lumen cannot be taken as conclusive evidence against a local role in the mucosa, since prostaglandins may be rapidly metabolized at their site of action, or may pass rapidly into the mucosal capillaries and be carried away by the increased mucosal blood flow.

The failure to detect prostaglandin output necessitated a study of the effects of indomethacin on mucosal prostaglandin activity, though it is not known how extractable prostaglandin reflects the activity of the prostaglandin system *in vivo*. Indomethacin markedly reduced the prostaglandin-like activity of mucosal extracts. Although some of the prostaglandin-like activity may have been formed *in vitro* prior to homogenization, the results indicate that the concentration of indomethacin in mucosal tissue *in vivo* was sufficient to inhibit prostaglandin synthesis. Inhibition of prostaglandin synthesis in human gastric mucosa *in vitro* by indomethacin has recently been reported (Bennett *et al.*, 1973).

We have confirmed that non-steroidal anti-inflammatory drugs such as indomethacin and phenylbutazone produce gastric mucosal erosions in the rat when administered parenterally. We have also shown that, at a time when the incidence and severity of mucosal erosions had reached a steady level, gastric mucosal prostaglandin levels were much reduced. The precise mechanism underlying the formation of erosions by aspirin-like drugs is unknown. Our results in the rat raise the possibility that a reduction of mucosal blood flow per unit acid secretion, possibly resulting in mucosal ischaemia, coupled with an increased sensitivity of the parietal cells to secretory stimuli, may contribute to the production of erosions. An increase in acid output is itself unlikely to cause erosions, but would promote their formation, as suggested by the increased incidence of mucosal bleeding following indomethacin at high rates of acid secretion. Thus, the prevention of indomethacin-induced mucosal erosions by prostaglandin E_2 and its (15S) methyl analogue (Main & Whittle, unpublished results), which are potent inhibitors of rat gastric acid secretion (Main & Whittle, 1974b), may reflect an antisecretory action rather than the role of endogenous prostaglandins. Whether the changes in mucosal permeability to ions, water and protein which often characterize mucosal damage by aspirin-like drugs (Davenport, 1964) can also be attributed to changes in mucosal prostaglandins has yet to be determined.

To establish causal relationships will require careful investigation not only of the relative potency of non-steroidal anti-inflammatory drugs in inhibiting gastric mucosal prostaglandin synthetase and in causing erosions, but of the simultaneous effects of these and other drugs which affect the prostaglandin system on gastric secretion, mucosal blood flow and mucosal permeability. In view of the species differences in susceptibility to erosion formation (Wilhelmi, 1974) and the possibility that differences exist between prostaglandin synthetase in different tissues (Flower & Vane, 1972) or in the same tissue from different species, it may be necessary to correlate these parameters in a single species.

Although the nature of the relationship between the intake of non-steroidal anti-inflammatory drugs and the incidence of peptic ulcer (Alp, Hislop & Kerr Grant, 1970) has not been clearly established, other factors which alter prostaglandin turnover or the relative amounts of different prostaglandins, may also be implicated in the pathogenesis of both acute erosions and peptic ulcer. It is also possible that drugs which are known to cause or exacerbate ulcers, for example the steroidal anti-inflammatory agents, may act by altering the prostaglandin system.

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INDOMETHACIN, KETOPROFEN AND CORPUS LUTEUM REGRESSION IN THE GUINEA-PIG

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1 The accelerated regression of the corpora lutea normally produced by glass beads inserted unilaterally into the uterus of the guinea-pig early in the oestrous cycle was prevented when the beads were filled with indomethacin or ketoprofen

2 Luteal regression was enhanced by the placement of beads in both horns of the uterus. When empty beads were placed in one horn of the uterus and drug-filled beads in the other, indomethacin blocked regression in both ovaries whereas ketoprofen was ineffective.

3 Indomethacin probably blocks luteal regression by inhibition of prostaglandin synthesis in the uterus.

Introduction

When glass beads are placed in the uterus of the guinea-pig early in the oestrous cycle regression of the corpora lutea is accelerated (Bland & Donovan, 1966). There is evidence that release of prostaglandin $F_{2\alpha}$ from the uterus late in the cycle brings about regression of corpora lutea and it seems likely that foreign bodies placed in the uterine lumen enhance the release of this compound (Poyser, Horton, Thompson & Los, 1970, 1971; Blatchley, Donovan, Horton & Poyser, 1972). In view of the reports that anti-inflammatory drugs such as aspirin or indomethacin inhibit prostaglandin synthetases (Vane, 1971), it was of interest to determine whether the luteolytic action of glass beads placed in the uterus could be blocked by indomethacin and ketoprofen, a chemically unrelated anti-inflammatory drug which inhibits prostaglandin synthetases. To ensure a high local concentration of drug the beads were packed with the compounds under test.

Methods

Adult guinea-pigs (475-1150 g) were used. The oestrous cycle was followed by daily inspection of the vagina and the collection of vaginal smears on those days when the vagina was open. Day 1 of the oestrous cycle was taken as the day when maximal cornification of cells was apparent before the post-ovulatory influx of leucocytes.

Glass beads were inserted into the uterus on days 3 or 4 as described previously (Donovan & Traczyk, 1962). The beads were cylindrical in shape, measuring approximately 7 x 3 mm and were sealed at one end. Immediately before insertion some beads were filled with drug by tamping. The surface of the packed down drug powder was thus accessible for solution in endometrial secretion through a caudally located orifice approximately 2 mm in diameter. The amount of indomethacin or ketoprofen packed into each bead was approximately 15 mg

The drugs used were indomethacin (Merck, Sharp & Dohme, Ltd.) and ketoprofen (May & Baker, Ltd.). In some experiments two beads filled with drug were placed in one uterine horn, and the other left undisturbed, in parallel experiments two beads filled with drug were placed in one horn and two empty beads in the other. In this way each animal could serve as its own control. The insertion of drug laden beads was alternated between the right and left uterine horns in the guinea-pigs subjected to each mode of treatment. For control purposes two empty glass beads were inserted into each uterine horn in a further group of animals.

All animals were killed on day 12 of the oestrous cycle, eight or nine days after operation, when the genital tract was removed and the two ovaries fixed separately. Serial sections of the ovaries were prepared and the sizes of the corpora lutea determined from measurement of two diameters at right angles in the plane of the largest section of each corpus luteum and multiplication of the product by the number and thickness of the sections. Animals in which corpora lutea were present in only one ovary were discarded, and sufficient animals prepared to bring the number of guinea-pigs in each group to ten. When the uteri were dissected some time after the experiments had been completed two empty beads were found to have been extruded from one uterine horn in an animal in which beads containing indomethacin were present on the opposite side. This group thus comprised nine animals.

Results

Luteal regression was inhibited when glass beads were filled with indomethacin before insertion into the uterus, when compared with the effect of empty beads (Table 1). With indomethacin present, there was no significant difference between the luteal volumes in the ovaries on the 'distended' or control sides.

Although the corpora lutea were also smaller on the treated side when ketoprofen-filled beads were inserted, the degree of luteal regression was not statistically significant ($P > 0.1$). Thus both drugs blocked luteal regression.

When two empty beads were inserted into each horn of the uterus, luteal regression was potentiated in both ovaries (Table 1). The mean luteal size following bilateral introduction of beads was 1.08 in the left ovaries compared with a figure of 2.05 for the corpora lutea on the treated side following unilateral insertion of beads. This difference is significant ($P < 0.01$).

Luteal regression was inhibited bilaterally when the beads inserted into one horn of the uterus were filled with indomethacin. Mean corpus luteum size on the treated side (2.95) was not significantly different from that of the control side (2.53), but considerably greater than that of animals provided with empty beads (2.95 *versus* 1.08; $P < 0.001$). The mean luteal size in the control ovaries of the indomethacin-treated animals (2.53) was also significantly greater than that for the ovaries (right, 1.07; left, 1.08) of the animals fitted only with empty beads ($P < 0.01$).

Ketoprofen did not inhibit luteal regression on the treatment side when beads were inserted bilaterally, the corpora lutea being similarly small in both ovaries (Table 1). The luteal bodies in the ovaries on the control or treated side of the animals given ketoprofen were not significantly larger than those of animals provided with empty beads in each horn ($P > 0.3$).

The total amount of indomethacin or ketoprofen contained in the beads in a uterine horn varied between 20 and 42 mg. When the uteri were dissected after preservation in formalin solution the beads packed with indomethacin were found still to contain substantial amounts of the drug, whilst the control bead and those originally filled with ketoprofen contained only fluid and a protein coagulum.

Macroscopic amounts of decidual tissue were infrequent after bead insertion, and the incidence of decidua was not altered by the presence of indomethacin or ketoprofen.

Discussion

Luteal regression ensued in the associated ovary when glass beads were inserted into one uterine

Table 1 The effect of indomethacin and of ketoprofen upon the luteal regression induced by the insertion of glass beads into the uterus of the guinea-pig

	Mean size of corpora lutea in the ovary associated with			
	Control horn	*Treated horn*	*Mean difference*	*Significance of difference*†
Unilateral bead insertion				
Empty beads*	3.74 ± 0.33	2.05 ± 0.33	1.69	$P < 0.001$
Indomethacin-filled beads	3.90 ± 0.28	3.45 ± 0.29	0.45	$0.2 > P > 0.1$
Ketoprofen-filled beads	3.54 ± 0.26	2.71 ± 0.47	0.83	$0.2 > P > 0.1$
Bilateral bead insertion				
Empty beads in each horn	1.07 ± 0.15	1.08 ± 0.13	0.01	$P > 0.9$
Indomethacin-filled beads in treated horn	2.53 ± 0.52	2.95 ± 0.54	0.42	$P > 0.5$
Ketoprofen-filled beads in treated horn	1.22 ± 0.38	1.30 ± 0.16	0.08	$P > 0.6$

* From Bland & Donovan, 1970.
† Paired *t* tests.

horn of the guinea-pig, but the degree of regression was greater when beads were introduced bilaterally. This observation indicates that the luteolytic factor produced in one uterine horn, while primarily influencing luteal function in the neighbouring ovary, can also affect the opposite gonad. Both indomethacin and ketoprofen could block the luteolytic response to unilateral insertion of glass beads, and these findings are in accord with the concept that luteal regression is mediated by the release of prostaglandin from the uterus.

Indomethacin also inhibited luteal regression on the control side when foreign bodies were placed in both uterine horns Thus it appears that sufficient indomethacin was released from the beads in one horn to affect prostaglandin synthesis in the whole of the uterus, despite the poor solubility of the drug. By contrast, ketoprofen did not influence luteal regression following the bilateral insertion of beads into the uterus. This finding could reflect a lesser potency of the drug in this experimental situation, particularly as ketoprofen was active when luteolysis was induced on one side only. No information is available concerning the rate, or time course, of release of indomethacin or ketoprofen from the interior of the beads. At autopsy, eight or nine days after insertion, solid ketoprofen was lacking inside the beads, but the drug content of the fluid present was not determined.

Indomethacin has extended the oestrous cycle of the guinea-pig when given systemically (Marley, 1972; Poyser, 1973), or by the intrauterine route contained in silicone rubber (Marley, 1973) or paraffin wax (Poyser, 1973; Horton & Poyser, 1973). Neither control cylindrical lengths of silicone rubber nor implants of paraffin wax caused a shortening of the oestrous cycle so that the endogenous production of prostaglandin appears not to have been enhanced by inert foreign bodies in these experiments The marked lengthening of the oestrous cycle with indomethacin observed by Horton & Poyser (1973) was accompanied by enlargement of the corpora lutea in the ovaries and the sustained secretion of progesterone.

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SYNTHESIS OF KALLIKREINS BY RAT KIDNEY SLICES

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1 Four radioactive kallikreins were isolated from rat kidney slices incubated with [^{3}H]-L-leucine

2 The kallikreins were purified by procedures previously used for the isolation of rat urinary kallikreins (Nustad & Pierce, 1974), and by affinity chromatography on a column of insolubilized anti-rat urinary kallikrein.

3 The kidney kallikreins resembled the urinary kallikreins in their relative amounts, isoelectric points and electrophoretic mobilities on polyacrylamide disc gels.

4 The data indicate that the kidney synthesizes four kallikreins which are released into urine The kallikreins are not changed by passage through the lower urinary tract.

Introduction

We have recently described the purification of four rat urinary kallikreins which were immunologically and biologically identical, but behaved differently in sodium dodecyl sulphate polyacrylamide disc gel electrophoresis and electrofocusing (Nustad & Pierce, 1974).

These findings and the preparation of specific antibody to the urinary kallikreins made it possible to test in a more definitive manner the hypothesis that urinary kallikrein is derived from the kidney (Werle & Vogel, 1960; Carvalho & Diniz, 1966; Nustad, 1970; Croxatto & Noe, 1972). The present study describes the isolation and characterization of kallikreins synthesized in rat kidney slices incubated with [^{3}H]-L-leucine, and the preparation and utilization of columns of purified anti-rat urinary kallikrein immobilized on agarose.

Methods

Preparation of immunoadsorbent

Purified sheep antibody to rat urinary kallikrein B_3 was obtained as described in Nustad & Pierce (1974) This procedure included immunoprecipitation of concentrated rat urine with the antiserum, dissociation of antigen and antibody by 8 M urea, and separation of antigen from antibody by gel filtration in 8 M urea on a Sephadex G-100 column. The purified antibody (100 A_{280} units of Fraction 1, Table II in Nustad & Pierce, 1974) was covalently bound to Sepharose 4B by the cyanogen bromide method (Axén, Porath & Ernback, 1967), with a 93% yield

Incubation of kidney tissue with [^{3}H]-L-leucine and preparation of soluble kallikrein

Sixty female Sprague-Dawley rats (200-300 g) were killed by a blow on the neck; the kidneys were removed aseptically and cut into slices (0.1-0.2 mm thick) with a Stadie-Riggs hand microtome (A.H. Thomas Co.). To each gram of tissue (wet weight) was added 10 ml of a medium consisting of: [^{3}H]-L-leucine-4,5 (2 μCi/ml), Eagle Basal Medium (minus L-leucine) with Hanks balanced salt solution (Merchant, Kahn & Murphy, 1964), and 0.05 M Tris HCl, pH 7.4 Before incubation the medium was gassed for 1 min with 95% O_2-5% CO_2. Following incubation in a shaking water bath for 1 h at 37°C, the slices were homogenized by 10 strokes of a Potter-Elvehjem homogenizer (1370 rev/min). An extract was prepared by treating the homogenate with desoxycholate (0.5% w/v), then centrifuging it at 48,000 *g* for 60 min and storing at −20°C. The supernatants from 6 incubates, each derived from the kidneys of 10 rats, were pooled, dialysed against 0.20 M KCl-0.01 M potassium phosphate, pH 7.0, and centrifuged at 48,000 *g* for 4 h to give Fraction 1 (Table 1).

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Colorimetric assay for α-N-carbobenzyloxy-L-arginine methyl ester (ZAME) hydrolysis

The purification of rat kidney esterases was followed using Roberts' (1958, 1960) method with a minor modification. The reaction between ZAME and 2 M alkaline hydroxylamine (NH_2OH HCl) was very rapid, maximum colour developed after 30-60 s incubation and then diminished. In contrast, the reactions of α-*N*-benzoyl-L-arginine ethyl ester (BAEE) and α-*N*-tosyl-L-arginine methyl ester (TAME) with alkaline hydroxylamine were complete in 1 and 12 min, respectively; the colour yield was not reduced after long incubation periods. To each test tube containing 0.20 ml of enzyme and 0.10 ml of 0.75 M Tris HCl, pH 8.5, was added 75 μl of 0.10 M ZAME. After incubation for 1.0 h at 37° C, the tubes were treated with 0.50 ml of freshly prepared 2.0 M NH_2OH . HCl-3.5 M NaOH and mixed. After 30 s, 0.25 ml of 0.37 M TCA-8.0 M HCl and then 4.0 ml of 0.11 M $FeCl_3$-0 04 M HCl, were added. The absorbance at 525 nm was determined 5 min later in a 1.0-cm cuvette. The amount of enzyme which hydrolyses one μmole of ZAME per min under these conditions was defined as one esterase unit (EU). A standard curve with ZAME was used to calculate the amount of ester hydrolysed by the enzyme preparations.

Conductivity determination

The conductivity of fractions from the DEAE-Sephadex column was determined in milliSiemens (mS) at 25° C, using a conductivity cell CDC 314 and conductivity meter CDM 3 (Radiometer).

Liquid scintillation counting

Disc gel sections of 10 to 60 mg (wet weight) were weighed in glass counting vials and dissolved by incubation with 0.1 ml of 30% hydrogen peroxide for 20 h at 50° C (Young & Fulhorst, 1965). The vials were cooled to room temperature, left without caps for 1 h, treated with 0.5 ml of Soluene, and incubated for 2 h at 50° C. The liquid scintillation fluid (10 ml per vial) contained: 400 g of naphthalene, 25 g of PPO, 0.25 g of POPOP, 1925 ml of dioxane, 1925 ml of xylol, and 1150 ml of absolute alcohol. Counting efficiency for tritium was 43%, using a Mark II Liquid Scintillation Counter (Nuclear Chicago).

Protein determination

This was measured by the adsorbance at 280 nm in a 1-cm cuvette, and in main fractions, also by the method of Lowry, Rosebrough, Farr & Randall (1951). Pure urinary kallikrein B_3 (Fraction 4c, Table I in Nustad & Pierce, 1974) was used as a protein standard.

The biological assay of kallikrein, phosphate determination, electrofocusing, and electrophoresis in 16.25% polyacrylamide disc gels are described in Nustad & Pierce (1974). The pressure dialysis of protein solutions and preparation of hydroxyapatite will be reported elsewhere.

Materials

The following were used in addition to the materials listed in Nustad & Pierce (1974), we obtained [^{3}H]-L-leucine-4,5 (15 Ci/mmole TRK 75) from Amersham/Searle, α-*N*-carbobenzyloxy-L-arginine methyl ester (ZAME) from Cyclo Chemical; sodium desoxycholate and bovine serum albumin (Fraction V) from Sigma Chemical Co., Sepharose 4B from Pharmacia; cyanogen bromide from Fluka, ultrapure sucrose from Mann; 2,5-diphenyloxazole (PPO) and 1,4-di(2-(5-phenyloxazolyl))-benzene (POPOP) from Koch-Light Lab., Ltd; Soluene 100 from Packard Instrument Co. and Aprotinin from Bayer, Leverkusen.

Results

Rat urinary kallikrein hydrolysed ZAME much more rapidly than the more commonly used substrates, TAME and BAEE (Nustad & Pierce, 1974). Consequently, hydrolysis of ZAME was used to follow the purification of kallikrein from kidney tissue, where the concentration of kallikrein is low and a sensitive assay is required (Nustad, 1970). The purest preparation of rat urinary kallikrein B_3 (23 TAME EU/mg) had a specific activity of 430 ZAME EU/mg; the purity of kallikrein preparations was therefore calculated assuming that pure enzyme should have a specific activity of 430 ZAME EU/mg. Kallikrein activity was also determined on the isolated rat uterus without addition of kininogen (Nustad & Pierce, 1974).

Testing of the immunoadsorbent

(a) *Rat urinary kallikrein* The capacity of the immunoadsorbent to bind rat urinary kallikrein was determined by passing a sample of pressure-dialysed and freeze-dried rat urine (233 mg of protein, calculated to contain 4 mg of pure kallikrein), in 6 ml of 0.15 M NaCl-0.01 M sodium phosphate, pH 7.4, over a 1.0 x 10-cm column of Sepharose 4B containing 36 mg of purified antibody. The column was washed with 130 ml of 0.15 M NaCl-0.01 M sodium phosphate, pH 7.4; 10 ml of 3 M NaCl-0.20 M sodium phosphate, pH 6.0, and 40 ml of 0.15 M NaCl-0 01 M sodium phosphate, pH 6.0. About one-half of the kallikrein (2 mg) in the starting sample was adsorbed on to the column. The molar ratio of antibody/bound antigen was calculated to be 3.7, whereas a ratio of 1.6 had been calculated for the immune precipitate from which the purified antibody had been obtained (Nustad & Pierce, 1974). The binding capacity of purified antibody on the column would then be 43% of that found for antibody in the antiserum.

The bound kallikrein was eluted with 8 M urea-0.15 M NaCl-0.01 M sodium phosphate, pH 6.0. After removal of urea by dialysis against 0.15 M NaCl-0.01 M sodium phosphate, pH 6.0, and concentration by pressure dialysis, 51% of the kallikrein activity which had been bound to the column was recovered. This compares well with a recovery of 45% from the immunoprecipitate (Table II in Nustad & Pierce, 1974), where kallikreins were recovered in two fractions after gel filtration in 8 M urea with purities calculated to be 42 and 82%, that is an average of 62%. The kallikreins recovered from the immunoadsorbent were 55% pure. Electrophoresis in 16.25% polyacrylamide gels showed one major band in the middle of the gel with faint bands on either side—a pattern indistinguishable from that found for the kallikreins isolated from the immunoprecipitate (Fraction 3 of Figure 5 in Nustad & Pierce, 1974).

(b) *Rat kidney kallikrein* A 50-ml extract (0.15 M NaCl-0.01 M sodium phosphate, pH 7.4) of kidney cortex from 10 rats (Fraction 1, Table 1) was analysed as described above with a similar immunoadsorbent column. As in the case of urinary kallikrein, 50% of the ZAME esterase activity was recovered after the removal of urea, but this preparation was less than 1% pure. Polyacrylamide disc gel electrophoresis showed many bands staining with Amido Black, none of which could be identified with kallikrein. It was therefore decided that the large-scale purification of kallikrein from incubates of rat kidney slices and $[^3H]$-L-leucine should follow the same procedure previously used to purify the urinary enzymes (Nustad & Pierce, 1974). The immunoadsorbent was reserved for the final purification step.

Purification of rat kidney kallikrein from tissue incubated with $[^3H]$-L-leucine

The purification of kidney kallikrein (Table 1, Figure 1) was carried out at room temperature (20-22° C) except for the electrofocusing step (4° C).

Step 1 Desoxycholate-solubilized kallikrein from the incubated kidney slices of 60 rats was prepared as described in methods. This extract (Fraction 1, Table 1) contained 4200 ct/min per mg of protein.

Step 2 Fraction 1 in 1.5 l of 0.20 M KCl-0.01 M potassium phosphate, pH 7.0, was adsorbed on to a DEAE-Sephadex A-50 column equilibrated with the same buffer. The column was developed as described in Figure 1a. All of the applied esterase activity was eluted as one peak at 40 mS, corresponding to 0.32 M KCl-0.01 M potassium phosphate, pH 7.0 (Figure 1a). The most active fractions were pooled, concentrated by pressure dialysis, and equilibrated against 3.0 M NaCl-0.01 M sodium phosphate, pH 6.0, to give Fraction 2.

Step 3 Fraction 2 in 25 ml of 3.0 M NaCl-0.01 M

Table 1 Purification of kallikreins synthesized in kidney slices from 60 rats

Fraction	*Treatment*	*Protein (mg)*	*Specific activity (EU*/mg)*	*Purification*	*Recovery (%)*
1	Rat kidney extract	3600	0.05	(1)	(100)
2	DEAE-Sephadex A-50 chromatography of Fraction 1	414	0.34	7	82
3	Hydroxyapatite chromatography of Fraction 2	37	2.2	46	47
4	Electrofocusing of Fraction 3	17	3.8	80	38
5	Affinity chromatography of Fraction 4	0.75	33.0	700	15

* 1.0 EU (esterase unit) = 1.0 μmole of ZAME/min (pH 8.5, 37° C).

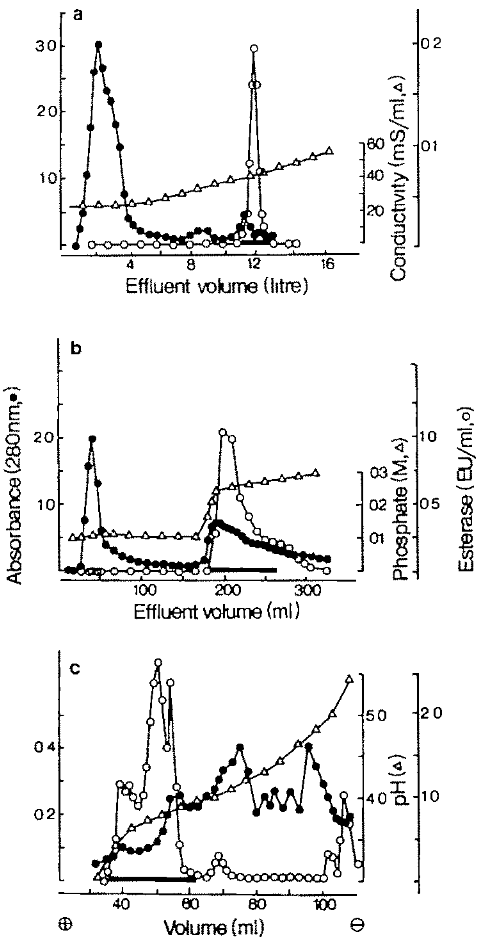

Figure 1 (a) DEAE-Sephadex A-50 chromatography. Column. 5 x 74 cm. Starting sample· 3600 mg of Fraction 1 (Table 1) in 1.5 l of the equilibration buffer (0.20 M KCl-0.01 M potassium phosphate, pH 7.0) Eluents. 4.0 l of the equilibration buffer; 10.0 l of a linear gradient from the equilibration buffer to 0.50 M KCl-0.01 M potassium phosphate pH 7.0 Fraction volume. 20 ml Flow rate: 40 ml/hour. Inside right ordinate. conductivity (mS/ml, △). (b) Hydroxyapatite chromatography Column· 1.25 x 29 cm. Starting sample 414 mg of Fraction 2 (Table 1) in 25 ml of the equilibration buffer (3 M NaCl-0.01 M sodium phosphate, pH 6.0). Eluents 110 ml of the equilibration buffer; 800 ml of a gradient from the equilibration buffer to 3 M NaCl-0.05 M sodium phosphate, pH 6.0 Fraction volume. 4.0 ml. Flow rate· 3.5 ml/hour. Inside right ordinate phosphate (M, △). (c) Electrofocusing. Column 110 ml containing 0.8% Ampholine, pH 3-5. Starting sample. 37 mg of Fraction 3 (Table 1) in 3.3 ml of 0.01 M sodium phosphate, pH 7.0. Operation: 400 → 1000 V/5 h, 1000 V/13 hour. Fraction volume. 1 ml. Elution flow rate: 120 ml/hour. Inside right ordinate· pH (△).

sodium phosphate, pH 6.0, was adsorbed to a hydroxyapatite column and was developed as described in Figure 1b. All of the esterase activity was eluted as one peak at a phosphate concentration of 0.024 M The most active fractions were pooled, concentrated by pressure dialysis, and equilibrated against 0.01 M sodium phosphate, pH 7.0, to give Fraction 3.

Step 4 Ultrapure sucrose (2.15 g) and 40% Ampholine pH 3-5 (0.15 ml) were added to ice-cold Fraction 3 in 3.3 ml of 0.01 M sodium phosphate, pH 7.0, and made to 8.8 ml with distilled water. This fraction replaced those tubes in the stepwise gradient with which it was isodense. Five esterase peaks with kallikrein activity were obtained with isoelectric points of 3.52, 3.65, 3.74, 3.80, and 4.0 (Figure 1c). The four kallikrein peaks with isoelectric points of 3.52 to 3.80 were pooled, dialysed against 3.0 M NaCl-0.01 M sodium phosphate, pH 6.0, and concentrated by pressure dialysis while equilibrating against 0.15 M NaCl-0.01 M sodium phosphate, pH 7.4, to give Fraction 4. The fifth kallikrein with pI 4.0 was not used since preliminary experiments indicated that it probably arose from the other four kallikreins by minor bacterial action

Step 5 Fraction 4 in 50 ml of 0.15 M NaCl-0.01 M sodium phosphate, pH 7.4, was applied to a 1.0 x 4.0-cm column of immobilized antibody to rat urinary kallikrein. The column was then washed with 115 ml of 0.15 M NaCl-0.01 M sodium phosphate, pH 7.4, 16 ml of 3.0 M NaCl-0.20 M sodium phosphate, pH 6.0; and 50 ml of 0.15 M NaCl-0.01 M sodium phosphate, pH 6.0 No esterase activity appeared in the effluent, the last 20 ml of which had an $A_{280\,\mathrm{nm}}$ of less than 0.015 and no radioactivity. The column was then eluted with 50 ml of 8.0 M urea-0.15 M NaCl-0.01 M sodium phosphate, pH 6.0. A small protein peak with total $A_{280\,\mathrm{nm}}$ of 0.34 was collected in 3 ml, dialysed against 0.15 M NaCl-0.01 M sodium phosphate, pH 6.0, and concentrated by pressure dialysis to 1.7 ml (Fraction 5). Electrofocusing of 0.6 ml of this fraction in a pH 3-5 gradient showed an enzyme profile like that of Fraction 4.

Disc gel electrophoresis of purified $[^3H]$-rat kidney kallikreins

Samples of 180 µl of Fraction 5 were electrophoresed in 16.25% polyacrylamide disc gels. A gel stained with Amido Black showed impurities at the cathodic end of the gel, a major band with a mobility of 25% of that of the dye front, and a minor band with faint bands on either side with a

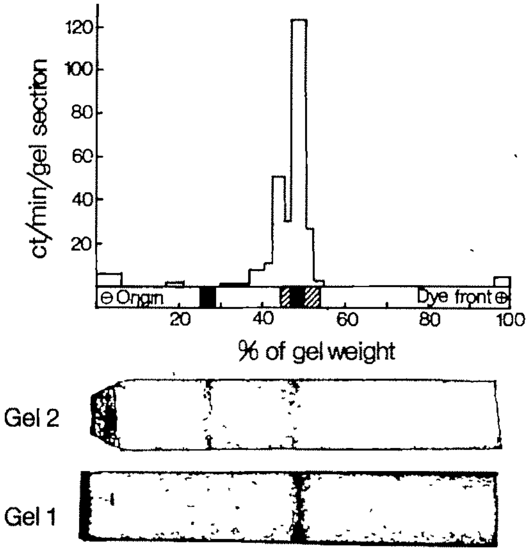

Figure 2 Electrophoresis of purified rat urinary and kidney kallikreins in 16.25% polyacrylamide disc gels at 20° C with 4 mA/tube for 2 hours Gel 1. 1.4 EU (3.3 μg protein) of rat urinary kallikrein B_3 (Fraction 4c, Table I in Nustad & Pierce, 1974.) Gel 2. 2.3 EU (67 μg protein) of rat kidney kallikreins (Fraction 5, Table 1). The figure above shows Gel 2 sectioned, dissolved in 30% hydrogen peroxide and counted for tritium.

mobility of 50% of that of the dye front (Figure 2).

An unstained gel (stored at −20° C) was sectioned, homogenized in 0.15 M NaCl-0 01 M sodium phosphate, pH 7.3, and assayed for esterase and kallikrein activities. Both activities were found in the same fractions with 78% and 64% recoveries, respectively, and corresponded exactly to the bands in the middle of the stained gel. The same correspondence was found for rat urinary kallikreins treated in the same way

The stained gel of Fraction 5 was sectioned, dissolved in hydrogen peroxide and counted. The radioactivity was recovered in 77% yield, in those sections from the middle of the gel where the kallikrein activity was found (Figure 2).

Bioassay of purified [^{3}H]-rat kidney kallikrein

The five esterases with pI's 3.50-4.0 gave contractions of the isolated rat uterus proportional to their esterase activities. The ability to contract the isolated rat uterus without addition of kininogen was previously found to be typical for rat urinary kallikreins (Nustad & Pierce, 1974). The oxytocic activity of each kallikrein was inhibited by antiserum to rat urinary kallikrein B_3 and by aprotinin, but not by soybean trypsin inhibitor

Discussion

The newly synthesized kallikreins isolated from kidney slices incubated with [^{3}H]-L-leucine were indistinguishable from kallikreins isolated from the urine of rats of the same strain The isoelectric points of the kidney enzymes—3 52, 3.65, 3.74 and 3 80—agree closely with those found earlier for the urinary enzymes—3.50, 3.68, 3.73 and 3.80. Furthermore, their relative amounts were similar.

The relative amount of enzyme activity under each peak of urinary kallikreins B_1-B_4 (Figure 1c in Nustad & Pierce, 1974) were 5, 12, 66 and 19%, whereas the corresponding figures for kidney kallikreins (Figure 1c) were 11, 15, 52 and 22%, respectively. The slight differences in the distribution of enzyme activities were probably due to the difference in the slopes of the pH gradient. Finally, the mobilities of the kidney and urinary enzymes in polyacrylamide gel electrophoresis were the same, and the active bands from the kidney contained almost all of the radioactivity of the applied sample. Besides indicating the identity of kidney and urinary kallikreins, these results show that kallikreins synthesized and released by the kidney are not changed during their passage through the lower urinary tract.

Kidney kallikreins presumably act on kininogen to produce kallidin (lysyl-bradykinin) in the kidney. Although the kininogen could come from the blood, there is some evidence that the kidney itself contains kininogen (Sardesai, 1968; Werle & Zach, 1970). Since kallikreins are synthesized in the kidney and free kinins are found in urine, the kallikrein-kinin system may participate in the local regulation of renal blood flow. This could be due to direct effects on vascular smooth muscles by kinins formed in the intercellular space. However, an indirect action of kinins on renal haemodynamics could be mediated through release of prostaglandins in the medulla by kinins formed in tubular urine (McGiff, Terragno, Malik & Lonigro, 1972; Stoner, Manganiello & Vaughan, 1973; McGiff, Crowshaw & Itskovitz, 1974). A role of kinins in the regulation of sodium excretion was suggested by Adetuyibi & Mills (1972) and Marin-Grez, Cottone & Carretero (1972). A role in the general regulation of blood pressure should also be considered, in view of several reports of decreased urinary kallikrein excretion in patients with essential hypertension (Elliot & Nuzum, 1934; Werle & Korsten, 1938; Margolius, Geller, Pisano & Sjoerdsma, 1971; Chernova &

Nekrasova, 1971, Margolius, Geller, de Jong, Pisano & Sjoerdsma, 1972).

The present study, demonstrating the synthesis of kallikreins in the kidney and their apparent identity with the urinary enzymes, contributes to the growing evidence for a physiological role of the kinin system.

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THE DISTRIBUTION OF LITHIUM, SODIUM AND MAGNESIUM IN RAT BRAIN AND PLASMA AFTER VARIOUS PERIODS OF ADMINISTRATION OF LITHIUM IN THE DIET

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1 The tissue solubilizer Soluene-100 provides an efficient and easy means of preparing small amounts of rat tissue for cation analysis.

2 Administration of lithium ions to rats for two days to 42 days by the addition of lithium chloride to the diet at a concentration of 30 mmol/kg dry weight results in (a) the uniform distribution of lithium throughout the brain at a concentration comparable to that found in plasma; (b) decrease in the brain sodium concentration; (c) a decrease in brain magnesium concentration and an increase in plasma magnesium concentration; (d) no change in brain water content.

3 The inclusion of LiCl in the diet at a concentration of 30 mmol/kg dry food gives consistent and predictable plasma and brain levels of lithium in the rat without the occurrence of serious side effects over periods of up to 42 days.

Introduction

Interest in lithium as an agent for the treatment of the affective disorders has grown steadily over the past few years. However, despite its intensive use, little is known of the biochemical basis for its behavioural effects. For the interpretation of biochemical and behavioural studies of lithium action, a knowledge of the distribution of lithium ions and other cations in the brain is desirable.

In previous studies various methods of administration have been used, including intravenous (Schou, 1958), intraperitoneal and oral by stomach tube (Morrison, Pritchard, Braude & D'Aguanno, 1971), and by adding lithium to the drinking water (Birch & Jenner, 1973). The consistent finding in whole brain studies was that lithium entered the brain more slowly than it did most other tissues. However, the concentration ratio between plasma and brain depended on the route and duration of administration, as well as the nature and amount of the lithium salt given.

Some workers have examined the distribution of lithium in the brain and its effect on the concentrations of other electrolytes (Ho, Gershon & Pinckney, 1970; Edelfors & Gøthgen, 1971). A higher concentration of lithium was found in the hypothalamus.

This paper describes the development of a simple assay for cations in small amounts of rat tissue. The method to be described uses Soluene-100 (Packard Scientific), a quaternary ammonium base, to solubilize tissues. The use of this agent has previously only been described for the estimation of zinc, copper, iron and manganese (Jackson, Michael & Schumacher, 1972). This new method of assay has been used to study the distribution of lithium, magnesium and sodium in rat brain after administration of lithium in the diet for periods ranging from 2 to 42 days.

Methods

Animals

The animals used were male CFY strain Wistar rats (Carworth Europe) which were 28 days old at the beginning of each experiment. They were allowed to acclimatize to laboratory conditions for one week. During this time they were kept three to a cage with a 12 h light-dark cycle (LD 12 : 12) (lights on 08 h 00 min-20 h 00 min) and given food (Carworth Dixon C-DD-R) and distilled water *ad libitum*.

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Management

After acclimatization the conditions were kept constant but lithium or sodium was added to the food. A slurry of 50% ground food pellets and 50% distilled water containing either sodium chloride or lithium chloride at concentrations of 30 mmol/l or 45 mmol/l was then given *ad libitum*, and replenished every other day. Daily intake of food and water was measured for each group of rats on alternate days, and the individual body weights were recorded. Treatment groups were as follows:

(1) Lithium chloride 30 mmol/kg dry food for 2 days (Li 30 2 days)
(2) Lithium chloride 30 mmol/kg dry food for 14 days (Li 30 14 days).
(3) Lithium chloride 30 mmol/kg dry food for 42 days (Li 30 42 days).
(4) Lithium chloride 45 mmol/kg dry food for 14 days (Li 45 14 days).

The controls for each of the groups consisted of rats given sodium chloride in the diet at the same molar dose and for the same length of time as the lithium chloride. A second control group given a normal diet (no additional sodium) was also included for use in the brain sodium concentration measurements.

Tissue preparation

At the end of the treatment period all animals were killed by cervical dislocation between 10 h 00 min and 11 h 00 min. The neck vessels were severed and blood collected in heparinized tubes. The brains were immediately dissected out (except for the olfactory lobes), rinsed with distilled-deionized water to remove extraneous matter and as many surface blood vessels as possible, and blotted dry. They were then dissected into seven regions following the scheme of Glowinski & Iversen (1966), except that a circular cut was made around the hypothalamus to reduce contamination with cortical tissue.

The blood collected from each rat was separated by centrifugation and the plasma stored at −20° C until assayed.

Each brain region from each rat was placed in a tared vial with an air-tight closure and weighed. Soluene-100 was added to the vial to give an approximate 10-fold tissue dilution. The tissue was left to solubilize for 24 to 48 h at room temperature. Immediately before assay all the solubilized samples were diluted approximately three-fold with 2-ethoxyethanol and weighed. The exact dilution of the tissue sample was calculated on a weight for weight basis. The same method was used to prepare whole-brain samples for assay. Cortical sections from an additional group of rats similar in all respects to Li 30 14-day rats but killed at 19 h 00 min were also prepared in the same manner.

The wet ashing method described by Chang, Gover & Harrison (1966) was used to prepare one set of cortex samples.

Assay

All samples treated with Soluene were assayed using a Hilger-Watt Atomspek AA2 atomic absorption spectrophotometer. Lithium assays were carried out with instrument settings recommended by the manufacturer for lithium, except that the burner height was increased to 5 mm and the air flow was increased by 10%.

A tissue blank prepared from the brain of a control animal treated as described above, and a reagent blank consisting of one part Soluene-100 to two parts 2-ethoxyethanol, were analysed with each batch of samples. Lithium standards were prepared by adding aqueous lithium chloride solutions to the reagent blank.

Samples of plasma, and aqueous samples obtained by the wet ashing method, were assayed for lithium using the instrument settings actually recommended by the manufacturer for lithium. Before assay all aqueous samples were diluted one part to three parts with a solution containing 6.5 g $La(NO_3)_3$, 0.67 g CsCl and 10 ml conc. HCl per litre of distilled water.

Sodium and magnesium determinations were carried out on aliquots of the diluted solubilized samples further diluted one part to ten parts and one part to four parts with 2-ethoxyethanol respectively. To the samples for magnesium determinations HCl and $La(NO_3)_3$ were added to a final concentration of 0.1N and 0.1%, respectively. Internal standards were used for both ions and instrument settings were those recommended by the manufacturer for sodium and magnesium.

Brain water content

Animals of the same type as those used for the cation analysis were given lithium chloride or sodium chloride in the food at a dose level of 30 mmol/kg of dry food for 14 days. At the end of this period they were killed and the brains were dissected to give the seven regions. Each region was weighed and then dried by heating at 110° C to constant weight.

Results

The analytical method used here has been compared with the standard technique of wet ashing as described by Birch & Jenner (1973) based on the procedure of Chang *et al.* (1966). The results of estimations of lithium, sodium and magnesium obtained by the two methods on similarly treated animals were not significantly different ($P > 0.05$, *t* test). When lithium is administered in the diet at a concentration of 30 mmol/kg of dry diet, an even distribution of lithium throughout the brain is reached within two days and remains essentially unaltered for up to 42 days. The values obtained after 14 days are representative of the entire experimental period (Table 1). Some of the brain regions show statistically significant differences in lithium concentrations after the various durations of administration; all values fall within a narrow range, however, and it is not possible to decide whether these differences have any real biological significance.

When the lithium dose was increased to 45 mmol/kg dry food, the brain region distribution of lithium remained similar but the concentrations increased (Table 1). However, the brain/plasma lithium ratio increased so that the concentrations of lithium in the hippocampus, mid-brain, corpus striatum and cortex were all significantly higher than the plasma lithium concentrations. The only exception to this was the hypothalamus where the ratio remained constant.

The whole brain concentration of lithium after the 30 mmol dosage level for 14 days is not significantly different from the plasma concentra-

Table 1 Rat brain and plasma concentrations of sodium, magnesium and lithium after the administration of a normal diet or diets with added NaCl (30 mmol/kg dry food) or LiCl (30 mmol or 45 mmol/kg dry food) for 14 days

	Normal diet	*Sodium diet NaCl 30 mmol/kg dry food*		*Lithium diet LiCl 30 mmol/kg dry food*			*Lithium diet LiCl 45 mmol/kg dry food*
	Na	Na	Mg	Na	Mg	Li	Li
Pons and medulla	51.7 ±0.91 (6)	52.8 ±0.89 (10)	5.69 ±0.38 (10)	47.3‡ ±0.95 (10)	5.03 ±0.24 (10)	0.49* ±0.021 (12)	0.74 ±0.029 (6)
Cerebellum	52.7 ±0.96 (6)	51.7 ±0.79 (10)	5.61 ±0.24 (10)	48.9‡ ±1.46 (10)	4.42‡ ±0.14 (10)	0.46* ±0.025 (12)	0.79 ±0.031 (6)
Hypothalamus	52.8 ±0.77 (6)	53.1 ±0.92 (10)	6.11 ±0.37 (6)	48.7‡ ±1.68 (10)	4.35 ±0.40 (6)	0.48* ±0.017 (12)	0.63* ±0.037 (5)
Hippocampus	52.5 ±1.10 (6)	52.8 ±1.46 (10)	5.86 ±0.37 (6)	52.1 ±0.92 (10)	4.62‡ ±0.38 (6)	0.61 ±0.023 (12)	1.0* ±0.047 (6)
Mid-brain	54.7 ±0.58 (6)	55.1 ±0.89 (10)	5.28 ±0.33 (10)	52.6‡ ±0.85 (10)	4.58 ±0.20 (10)	0.55 ±0.021 (12)	0.90* ±0.023 (6)
Corpus striatum	51.3 ±0.96 (6)	51.5 ±1.61 (10)	7.41 ±0.36 (6)	49.6 ±0.85 (10)	4.49‡ ±0.39 (6)	0.64 ±0.021 (11)	1.16* ±0.051 (5)
Cortex	51.8 ±0.73 (6)	51.2 ±0.47 (10)	5.67 ±0.21 (10)	47.5‡ ±1.71 (10)	5.03‡ ±0.14 (10)	0.63 ±0.022 (12)	1.22* ±0.040 (5)
Cortex (19h 00min)	–	–	–	–	–	0.52† ±0.040 (5)	–
Whole brain	–	–	–	–	–	0.56 ±0.037 (6)	0.92 ±0.063 (6)
Plasma	–	–	0.69 ±0.03 (13)	–	0.90‡ ±0.03 (12)	0.59 ±0.020 (12)	0.79 ±0.031 (6)

Values are expressed as mmol/kg fresh tissue or as mmol/l of plasma and represent the mean ± s.e. mean. The number of observations is in parentheses. All values are from rats killed at 10h 00min unless otherwise stated.

* Significantly different from plasma concentration
† Significantly different from cortex (10h 00min) concentration } $P < 0.05$, *t* test
‡ Significantly different from Na diet concentration

tion and falls midway in the brain regions' lithium concentration range (Table 1) After the 45 mmol lithium dose the whole brain/plasma ratio of lithium concentration is increased and the whole brain lithium concentration again falls mid-way in the brain regions' range of lithium concentration.

The results show a small but significant drop in the lithium concentration in the cortex over the 9 h period from 10 h 00 min to 19 h 00 min. This period coincides with the normal light period for these animals (08 h 00 min to 20 h 00 min)

Unlike Edelfors & Gøthgen (1971), the present study revealed a significant decrease in the sodium content of all brain regions except the hippocampus and corpus striatum in rats given lithium for 14 days (Table 1).

The content of magnesium in animals treated with lithium for 14 days was decreased in all regions, reaching significance in all but the pons and medulla and mid-brain (Table 1). In accord with other studies (Birch & Jenner, 1973; Neilson, 1964a, b) in both man and animals, plasma magnesium was elevated in all the rats given lithium for 14 days.

Food intake for the first two days of lithium feeding was approximately 15% below control levels, but it increased to within 5% of control levels within the next four days. Weight increase of the lithium-treated rats was decreased for the first four days but returned to normal values over the rest of the experimental period.

Discussion

Pilot studies have shown that the Soluene method of tissue preparation is also suitable for K^+ determinations using a Technicon Auto Analyser and flame photometer. Calcium estimation on the other hand proved troublesome. This was due to precipitation problems which unlike those encountered during magnesium estimation could not be overcome by the addition of an acid. The limits of sensitivity of this method are set by the degree of dilution required to allow the sample to pass through the atomizer of the atomic absorption spectrophotometer.

These results differ in most respects from the only previous study which is at all comparable (Edelfors & Gøthgen, 1971) although few experimental details were given in that report which only appeared as an abstract. In those studies, following oral lithium administration for two or five weeks, comparatively high lithium levels were found in the hypothalamus compared with other brain regions. At the same time, the water to dry matter ratio decreased in all parts of the brain except the hypothalamus by about 50%. However, we found no significant change in water content of fresh brain tissue following lithium administration. Birch & Jenner (1973) also found no significant alteration of water content of whole brain after lithium administration.

Slight regional differences in water content were noted by us in both lithium- and sodium-treated animals. These were similar to those reported by Donaldson, St. Pierre, Minnich & Barbeau (1973), thus showing that any variation of the magnitude reported by Edelfors & Gøthgen (1971) should have been extremely obvious.

The absolute values of sodium and magnesium concentration in the brain regions of the sodium-treated control animals were very similar to those reported by Donaldson *et al.* (1973) using the same dissection technique but a dry ashing method for tissue preparation. Magnesium plasma levels in the sodium-treated controls were very similar to those reported by Mellerup, Plenge & Rafaelson (1973).

Comparing the results of the present study with those of Birch & Jenner (1973), both show a decrease in brain magnesium proportionally depending on the dose of lithium, although in the latter case lithium was administered by intraperitoneal injection or in the drinking water. Also, Mellerup *et al.* (1973) showed an increase in plasma magnesium after a single intraperitoneal injection of 900 μmol of lithium chloride which is comparable to the increased magnesium level we obtained with a very different dosage and route of administration.

It has been suggested (Baer, Kassir & Fieve, 1970) that the decrease in tissue sodium concentration in lithium-treated animals may only be apparent due to an elevated tissue sodium concentration in the controls if they are given extra sodium in the diet. However, the very close similarity in the sodium levels of the two non-lithium groups used here do not support this theory.

Many reports concerning tissue distribution of various ions after lithium administration have already been published (Schou, 1958; Ho *et al.*, 1970, Frazer, Mendels, Secunda, Cochrane & Bianchi, 1973). However, none have dealt with the regional distribution of sodium, magnesium and lithium in the rat brain after the administration of LiCl in the food at a dose producing a steady plasma/brain lithium ratio approaching unity, plasma levels within the human therapeutic range and no serious side effects when given for up to 42 days.

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VARIATION IN ACTIVITY OF MONOAMINE METABOLIZING ENZYMES IN RAT LIVER DURING PREGNANCY

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1 Catechol-*O*-methyltransferase (COMT) and monoamine oxidase (MAO) activities in rat liver were measured during pregnancy, parturition and postpartum. Compared with activity in non-pregnant controls, both enzymes showed a significant decrease in activity which was most pronounced at day 18.

2 The metabolism of intravenously infused [^{3}H]-adrenaline to [^{3}H]-metanephrine and to [^{3}H]-acidic metabolites was also significantly depressed during pregnancy but had returned to control values by the 21st day.

3 The effects of reserpine and/or nialamide on hepatic COMT and MAO were studied in control and 20-day-pregnant rats. Their action on COMT activity differed in the two groups. MAO was inhibited to a similar extent in these groups whether the drugs were given separately or in combination.

4 It seems possible that the changes in endocrine function which occur during pregnancy are responsible for the observed alterations in enzyme activity.

Introduction

The effects of certain sex hormones on catecholamine release and metabolism in adult animals of a number of different species are well established (Pohorecky & Wurtman, 1971; Parvez & Parvez, 1972a; Holzbauer & Youdim, 1973). Variations in release into the blood stream, in urinary excretion and in adrenal content of catecholamines have recently been observed during pregnancy (Parvez, Gripois & Parvez, 1973; Parvez, Parvez & Gripois, 1973a) when profound hormonal changes are known to occur. Earlier, Cohen, Bitensky & Chayen (1965), using histochemical methods, noted variations in monoamine oxidase (MAO) staining patterns in the human endometrium during the menstrual cycle: enzyme activity appeared weak and confined to intercellular particles early in the proliferative phase, becoming progressively more intense and diffuse later in the cycle. Shortly before the onset of menstruation the particulate appearance was superseded by a diffuse staining of the whole cell. Quantitative evidence of these induced changes in MAO activity was later obtained by biochemical assay (Southgate, Grant, Pollard, Pryse-Davis & Sandler, 1968). Approximately ten-fold increases were observed on day 21 compared with earlier in the cycle. Variations in the MAO activity during the oestrus cycle have been noted in certain rodents' organs, including brain (Zolovick, Pearse, Boehlke & Eleftheriou, 1966; Cavanaugh & Zeller, 1967; Kamberi & Kobayashi, 1970; Holzbauer & Youdim, 1972).

The present experiments were designed to study the activity of the enzymes MAO and catechol-*O*-methyltransferase (COMT) in rat liver at different periods during the second half of pregnancy, at parturition and postpartum. In addition, three groups of rats were injected with reserpine and nialamide on the 20th day of pregnancy and the MAO and COMT activities in their livers were compared with those of non-pregnant females. The accumulation of injected [^{3}H]-adrenaline and its metabolism in pregnant rats were also studied.

Methods

Female rats (Sherman strain, 12-15 weeks old, 300 ± 50 g) were used. They were housed at a constant temperature (23°C) with natural day and night cycle and fed with commercial laboratory food *ad libitum*. For fertilization, one male was kept with seven females from 19 h 00 min to 09 h 00 minutes. Pregnancy was verified by palpation 14 days later. Parturition occurred between day 21 and 22. For MAO and COMT analyses, animals were killed by neck fracture at 14 h 00 min on the specified day of pregnancy. Their livers were excised and kept in 0.9% w/v NaCl solution (saline) at −30° C.

[^{14}C]-Methyl-S-adenosyl methionine (sp. act. 50 mCi/mM) and [^{3}H]-(±)-adrenaline (sp. act. 4 Ci/mM were supplied by CEA, Gif sur Yvette, France. Tryptamine-[^{14}C]-bisuccinate (sp. act. 8.9 mCi/mM) was purchased from New England Nuclear Corporation, Boston, U.S.A. Normetanephrine (Calbiochem) and (±)-adrenaline (Rhone-Poulenc) were brought from Prolabo, Paris, France. Reserpine (Serpasil, Ciba) ampoules of 1 ml containing 2.5 mg suitable for subcutaneous administration, was supplied by Ciba Laboratories, Basel. Nialamide (in form of chlorohydrate, Pfizer Clinical Laboratories, Paris, France) was dissolved in sterilized olive oil. Control, as well as pregnant rats, were injected subcutaneously with reserpine (2.5 mg/kg) or nialamide (200 mg/kg). The doses for the combined administration were reserpine (1 mg/kg) + nialamide (100 mg/kg). In the pregnant rats, drugs were injected at 14 h 00 min on day 20 of pregnancy. This time was chosen to avoid stress during the last day of pregnancy or parturition.

Preparation of tissue for enzyme assay

Livers were homogenized in 40 volumes of ice-cold 0.9% KCl solution with glass homogenizers. Each homogenate was divided into two parts, one being stored for a short time at 1° C prior to MAO assay. The other was centrifuged for 30 min (50,000 *g*, 1° C) and the supernatant taken for COMT assay. Portions of homogenate and supernatant were boiled for 5 min and used as blanks.

Assay of MAO

The substrate used was tryptamine-[^{14}C]-bisuccinate (sp. act. 8.9 mCi/mM; Wurtman & Axelrod, 1963). The incubation mixture consisted of 0.44 nmole radioactive substrate in a volume of 50 µl, of 0.2 M phosphate buffer, pH 7.4, (200 µl) and tissue homogenate (100 µl). Otherwise, the procedure was as described (Wurtman & Axelrod, 1963) except that the final extraction was made into 3.5 ml toluene instead of six millilitres.

Assay of COMT

COMT was assayed using S-adenosyl methionine-[^{14}C] as methyl donor to one of the phenolic hydroxyl groups of adrenaline (Axelrod, 1962). The reaction mixture was prepared in glass tubes and consisted of 0.2 M phosphate buffer pH 7.9 (200 µl) 1% Mg Cl_2 (10 µl) 0.5 nmole adrenaline (50 µl), 0.44 nmole S-adenosyl methionine-[^{14}C] and enzyme preparation (100 µl). The tubes were closed and incubated in a water bath (37° C) for one hour They were then cooled and 0.5 M borate buffer, pH 10 (0.5 ml), was added to stop the reaction Toluene: isoamyl alcohol 3 : 1 (v/v) (3.5 ml) was added to each tube. After shaking for 25 min, tubes were centrifuged and 3 ml organic phase transferred to a scintillation vial containing 10 ml 'triton-toluene mixture' for counting.

Determination of [^{3}H]-adrenaline and its metabolites

The contents of one ampoule containing 1 mCi [^{3}H]-adrenaline were diluted to 3.33 ml with double-distilled water containing 0.001 M HCl. On the specified day of pregnancy, 1 ml of 0.9% saline containing 60 µCi of amine was infused into a saphenous vein over a period of 1 min under light ether anaesthesia. Twenty min later, the rats were killed by neck fracture and the livers excised immediately and homogenized in 8 ml of an ice-cold mixture of 4% trichloroacetic acid + 1% EDTA. [^{3}H]-Adrenaline was isolated on acid activated aluminium oxide (Euler & Lishajko, 1961) and eluted with 0.25 N acetic acid (2 ml). After centrifugation, 0.5 ml eluate was added to 10 ml liquid scintillator and counted for 10 minutes. [^{3}H]-Metanephrine and [^{3}H]-acidic metabolites were isolated according to the techniques of Axelrod & Tomchick (1960) and Axelrod, Weil-Malherbe & Tomchick (1959).

Results

COMT & MAO activity during pregnancy

Figure 1 shows COMT activity in rat liver at several periods of pregnancy, at parturition and postpartum. At day 18, activity was 44% lower than in non-pregnant controls. On day 20 of pregnancy, it was 27% higher than on day 18. It fell again on day 21 and at time of birth and was still low a few hours postpartum.

Figure 2 shows hepatic MAO activity at different stages of pregnancy. Compared to non-pregnant controls, activity was decreased by 24% on the 15th and 50% on the 18th day of pregnancy. At the 20th and 21st day, activity again increased, whilst a slight decline in activity

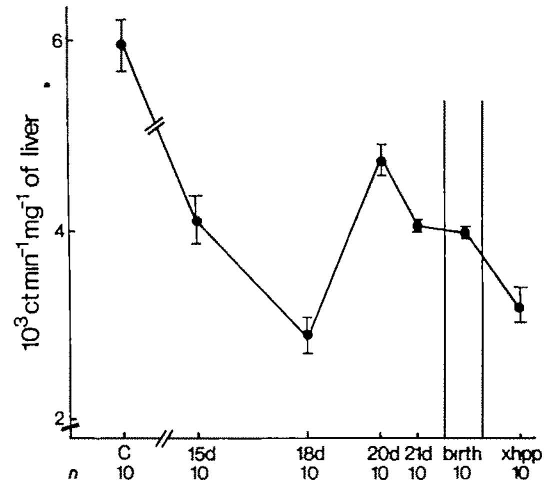

Figure 1 Catechol-*O*-methyltransferase (COMT) activity in rat liver during pregnancy, parturition and postpartum. *n* = number of animals used for each determination, C = non-pregnant females, 15d, 18d, 20d, 21d = days of pregnancy, and xhpp = 2 h after birth.

15d, 18d, 20d, 21d, birth and xhpp differ significantly from C($P < 0.001$) and 18d and 20d ($P < 0.001$).

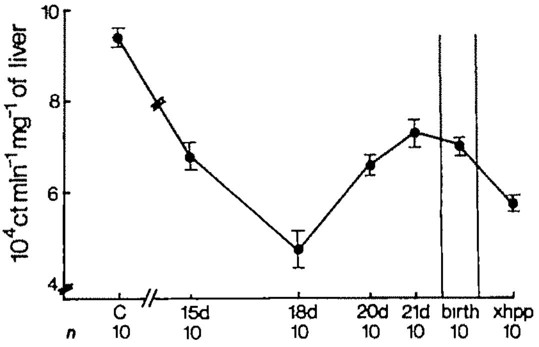

Figure 2 Monoamine oxidase (MAO) activity in rat liver during pregnancy, parturition and postpartum. *n* = number of animals used for each determination; C = non-pregnant females; 15d, 18d, 20d and 21d = days of pregnancy and xhpp = 2 h after birth.

15d, 18d, 20d, birth and xhpp differ significantly from C($P < 0.001$).

was observed during parturition which persisted for several hours afterwards.

The degree of degradation of intravenously infused $[^3H]$-adrenaline to $[^3H]$-metanephrine and $[^3H]$-acidic metabolites in male rats and in pregnant and non-pregnant females is compared in Figure 3. The amount of $[^3H]$-metanephrine and $[^3H]$-acidic metabolites found in the liver 20 min after an intravenous infusion of adrenaline was similar in males and in non-pregnant females. It was significantly lower in 18-day pregnant rats, but in 21-day pregnant rats was similar to control values.

Figure 4 provides a comparison between the effect of separate or combined administration of reserpine and nialamide on hepatic COMT activity in controls and in 20 days pregnant rats. In controls, reserpine inhibited COMT activity by 47%. In combination with nialamide, the same dose of reserpine did not produce any significant changes in COMT activity in pregnant rats. Nialamide decreased COMT activity in control and in pregnant rats by 23% and 40% respectively. The combined administration of reserpine and nialamide to non-pregnant females decreased COMT activity by 40%. However, similar treatment of 20-day pregnant rats produced only a 24% inhibition of COMT.

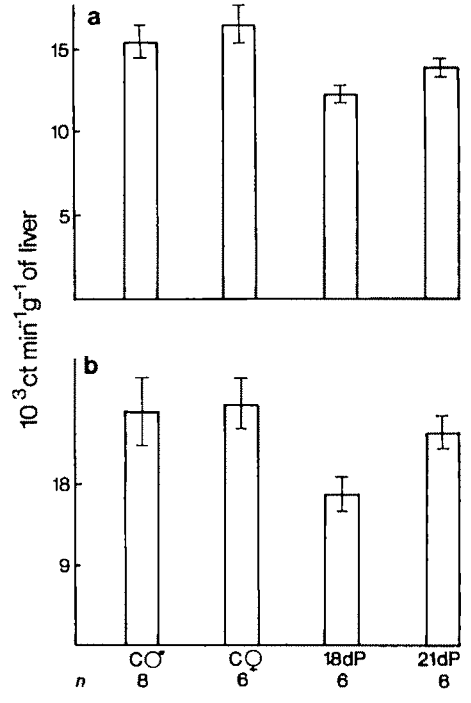

Figure 3 The transformation of $[^3H]$-adrenaline to (a) $[^3H]$-metadrenaline and (b) $[^3H]$-acidic metabolites in control male, female, 18 day pregnant (18dP) and 21 day pregnant rats (21dP). The animals received 60 μCi of tracer intravenously and livers were excised 20 min later.

Significant differences between female controls and 18dP were observed for metanephrine ($P < 0.01$) and acidic metabolites ($P < 0.01$).

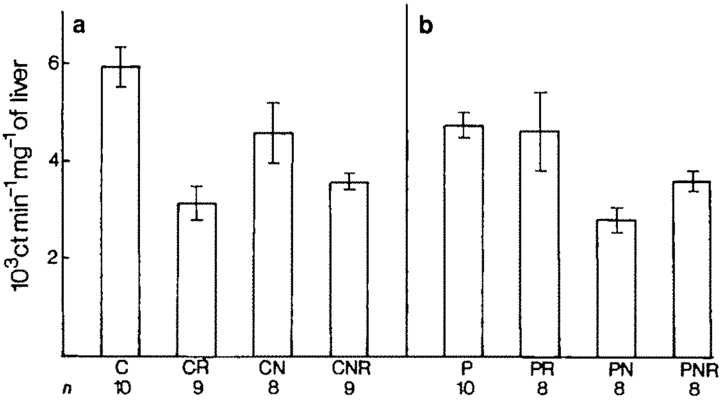

Figure 4 The effect of single or combined administration of reserpine (R) (2.5 mg/kg) and nialamide (N) (200 mg/kg) in (a) non-pregnant control (C) or (b) pregnant rats (P, 20 days) on catechol-*O*-methyl transferase (COMT) activity. *n* = number of rats.

Significance of differences between points:

C vs CR: $P < 0.001$; C vs CNR: $P < 0.001$; P vs PR: not significant; P vs PNR: $P < 0.01$; P vs PN: $P < 0.01$.

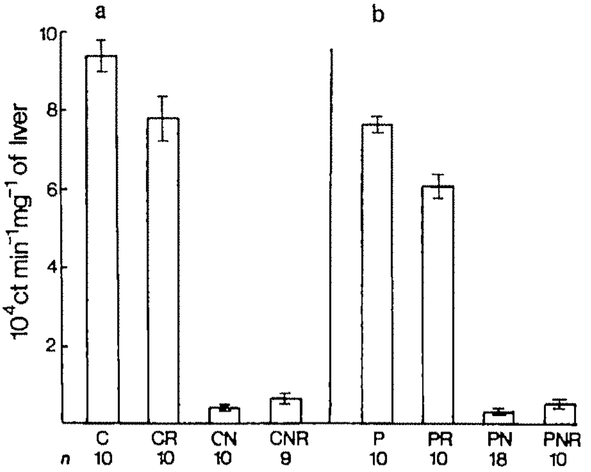

Figure 5 Monoamine oxidase (MAO) activity in (a) control (C) and (b) 20 day pregnant (P) rats after administration of reserpine (R) (2.5 mg/kg) or nialamide (N) (200 mg/kg). *n* =,number of rats.

Significance of differences between points:

C vs CR = not significant; P vs PR = not significant.

Figure 5 shows the effects of separate or combined administration of reserpine and nialamide on hepatic MAO activity in control and pregnant females. A single injection of reserpine (2.5 mg/kg) inhibited MAO activity by 17% in controls and 21% in pregnant rats. The administration of nialamide gave rise to inhibition of similar degree in both groups. Combined treatment resulted in 93% inhibition of MAO activity in both non-pregnant and pregnant rats, compared with untreated controls.

Discussion

The present results on pregnant rats provide presumptive evidence for hormones having an effect on catecholamine metabolizing enzymes. The decrease in activity of the two enzymes measured may conceivably be due to variation in the secretion of different hormones which are known to affect catecholamine metabolism. Evidence is available that corticosteroids and oestrogens inhibit catecholamine catabolism

(Callingham & Della Corte, 1972; Parvez & Parvez, 1972b, 1973; Holzbauer & Youdim, 1973). The naturally occurring changes in progesterone concentrations during the oestrous cycle are sufficiently large to influence MAO activity in various tissues including brain (Kamberi & Kobayashi, 1970; Holzbauer & Youdim, 1972; Youdim, Holzbauer & Woods, 1974). Some measure of the connection between MAO activity and progesterone production was provided by Holzbauer & Youdim (1973) who measured the progesterone content of ovaries and adrenal glands which synthesize and secrete about equal amounts of progesterone in the rat (Fajer, Holzbauer & Newport, 1971; Holzbauer & Godden, 1974). The extensive experiments of Ball, Knuppen, Haupt & Breuer (1972) who investigated the physico-chemical interactions between oestrogens and catecholamines and their effect on methylation of catechol oestrogens, catecholamines and other catechols by COMT, point to an important role for oestrogens in the control of amine metabolism.

Previous studies on the urinary excretion of vanilmandelic acid during pregnancy and parturition (Parvez, Gripois & Parvez, 1973) are compatible with our present findings on MAO and COMT activities. Fluctuations in vanilmandelic acid excretion run parallel to MAO and COMT activities on the 18th, 20th and 21st day of pregnancy. Maternal levels of corticosteroid and oestrogens determined throughout pregnancy (Milkovic, Milkovic & Paunovic, 1973; Challis, Heap & Illingworth, 1973) also tend to agree with the present results. Urinary output, and release into the blood steam of both adrenaline and noradrenaline is persistently increased during the last days of pregnancy (Parvez *et al.*, 1973a and b). When MAO and COMT activities are low, they may well mirror a decrease in catecholamine catabolism during pregnancy. Preliminary results on the maintenance of glycogen stores in late pregnancy in different organs of the rat contrast with the low hepatic glycogen of early pregnancy which may be related to the present findings (Parvez, Parvez & Youdim, unpublished observations).

The effect of reserpine and nialamide on hepatic MAO and COMT are consistent with the findings of others (Euler, Bygdemann & Pearsson, 1971; Spector, Tarver & Berkowitz, 1972); however, there were differences between control and pregnant rats which appear to be the consequence of hormonal state and suggest that varying concentrations of different hormones in pregnancy counter the effects of reserpine and nialamide. Fluctuating concentrations of progesterone, oestrogens and glucocorticoids during pregnancy are well documented. Our results suggest that these changes may have functional importance in controlling the production of biogenic amines by regulating the activities of their catabolizing enzymes.

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INFLUENCE OF COCAINE AND SODIUM ON BRETYLIUM UPTAKE BY RESERPINE-TREATED GUINEA-PIG LEFT ATRIUM

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1 The effects of cocaine and sodium on bretylium uptake into sympathetic nerve terminals were investigated in the reserpine-treated guinea-pig left atrium. The ability of bretylium pretreatment to increase the retention of noradrenaline was used as an index of bretylium uptake. Such increased retention has been assessed both by direct measurement and by the ability of tyramine to produce an inotropic response.

2 The restoration of the response to tyramine after incubation with noradrenaline was abolished when the atrium was pretreated with bretylium in the presence of cocaine. When bretylium was added before cocaine, or when α-methyl-noradrenaline (not a substrate for monoamine oxidase) was used for incubation, the responses to tyramine were restored in the normal way.

3 Bretylium greatly enhanced the retention of [^{3}H]-noradrenaline; when bretylium was added in the presence of cocaine, [^{3}H]-noradrenaline retention was severely impaired.

4 Pretreatment with bretylium in a low-sodium (25 mM) or sodium-free medium significantly decreased the retention of [^{3}H]-noradrenaline, as compared with the control.

5 Potassium deprivation did not modify the enhanced retention of [^{3}H]-noradrenaline induced by bretylium pretreatment.

6 Bretylium was released from the nerve terminals by exposure of the preparation to a sodium-free medium or to a solution containing calcium 50 mM, leading to a considerable decrease in [^{3}H]-noradrenaline retention.

7 The results are consistent with the view that both cocaine and sodium deprivation block the uptake of bretylium by the adrenergic nerve terminals, and that bretylium is probably taken up by a mechanism similar to or identical with the uptake system for noradrenaline and other amines.

Introduction

Boura & Green (1959) showed that bretylium interferes with the physiological release of noradrenaline from peripheral sympathetic nerve endings. Boura, Copp, Duncombe, Green & McCoubrey (1960) found that the adrenergic neuronal blocking action of bretylium was related to the concentration of this agent in postganglionic sympathetic nerve fibres. Later it was demonstrated that bretylium pretreatment increases the retention of exogenous noradrenaline by guinea-pig isolated left atrium, and enhances the ability of a brief incubation with noradrenaline to restore the inotropic response to tyramine in reserpine-treated guinea-pig left atrium (Furchgott, 1964; Furchgott & Sánchez-García, 1966; Furchgott, Sánchez-García, Wakade & Cervoni, 1971). On the basis of these results the authors suggested that bretylium behaves as an inhibitor of monoamine oxidase. Even though bretylium is a weak inhibitor of monoamine oxidase (McCoubrey, 1962; Kuntzman & Jacobson, 1963; Dvornik, Kraml, Dubuc, Tom & Zsoter, 1963) it enables effective monoamine oxidase inhibition in sympathetic nerves because of its selective accumulation in adrenergic neurones. Clarke & Leach (1968) reached similar conclusions based on the observation that treatment with bretylium potentiated the effect of infusions of noradrenaline and its precursors in restoring the cardiovascular responses to tyramine in rats pretreated with reserpine. Therefore, the prediction could be made that any agent or procedure able to interfere with bretylium accumulation in adrenergic neurones would inhibit, at the same time, its intraneuronal monoamine oxidase blocking activity.

The present study was undertaken in order to test the effects of cocaine and a low-sodium medium, which effectively block the uptake of noradrenaline (Furchgott, Kirpekar, Rieker & Schwab, 1963; Iversen & Kravitz, 1966), on the uptake of bretylium by the adrenergic nerve endings of atria from reserpine-treated guinea-pigs. The ability of bretylium pretreatment to increase the retention of noradrenaline in reserpinized atria has been used in the present work as an index of bretylium uptake. Such increased retention has been assessed both by direct measurement of $[^3H]$-noradrenaline and by the ability of tyramine to produce an inotropic response after a brief incubation with noradrenaline. A preliminary report of this work has already appeared (Sánchez-García, García & Velázquez, 1969).

Methods

Preparation of atria

Guinea-pigs weighing from 400 to 600 g were killed by a blow on the head after which their hearts were rapidly removed. The left atrium divided from base to tip into two halves was prepared for mounting as previously described by Furchgott *et al.* (1963). In a single experiment one half of the atrium served as a control and the other half as the experimental preparation The bathing medium was Krebs-bicarbonate solution having the following composition (mM): NaCl, 119; KCl, 4.7; $CaCl_2$, 2.5, $MgCl_2 . 7H_2O$, 1.18; $NaHCO_3$, 25; KH_2PO_4, 1.2; glucose, 10. The disodium salt of ethylenediaminetetracetic acid (EDTA) was always present in a concentration of 10 μg/ml in order to protect added noradrenaline from metal-catalysed oxidation. The solution was bubbled with 95% O_2 and 5% CO_2. When the sodium concentration was reduced the osmolarity of the solution was maintained with equivalent amounts of sucrose. High calcium (50 mM) solution was prepared by removing bicarbonate and adjusting the pH of the solution to 7.4 with 5 mM Tris-HCl buffer. Potassium-free Krebs solution was prepared by removing KH_2PO_4 and KCl. The muscle chamber was washed out with 200 ml of solution. All preparations were electrically driven (Grass Stimulator model SD. 5), at a frequency of 0.5 Hz and 5 ms duration. The resting tension applied to all preparations was 1 gram. Atria were attached to a force-displacement transducer (model FT. 03) and contractions were recorded by means of a Grass polygraph. Before drugs were added to the bath the atria were allowed to equilibrate for a period of 30 minutes. All experiments were performed at 37° C.

Incubation with and analysis for $[^3H]$-noradrenaline

In all experiments with $[^3H]$-noradrenaline the atria were incubated with a standard dose of (±)-$[^3H]$-noradrenaline (10 μCi total dose in a muscle chamber of 20 ml working volume). After exposure to $[^3H]$-noradrenaline for 5 min the preparations were washed with normal Krebs every 10 min, and finally removed for analysis 45 min after the first washout. Extraction of $[^3H]$-noradrenaline was performed according to the method described by Anton & Sayre (1962). The radioactivity present in the eluates from alumina was measured in a Packard Tri-Carb liquid scintillation counter Samples were prepared for counting by the addition of 1 ml of the eluate to 14 ml of the scintillation solution (Bray, 1960). Results are expressed as disintegrations per min per gram of tissue (d min^{-1} g^{-1} tissue), ± standard error of the mean. 'Noradrenaline uptake-retention' means noradrenaline taken up and retained in the tissue after the 45 min washout period. All values given for $[^3H]$-noradrenaline in this paper are corrected for recovery which was about 75%. The statistical significance of the difference between means was determined by Student's *t* test for paired or group data.

Reserpine treatment

Pretreatment with reserpine (5 mg/kg) was carried out by a single intraperitoneal injection 18 to 24 h before the experiments.

Drugs used

The drugs used were: (±)-noradrenaline-[7-^{3}H]-hydrochloride, specific activity 7 Ci/mmol (New England Nuclear Corp.); (−)-noradrenaline bitartrate; (±)-α-methyl-noradrenaline hydrochloride; tyramine hydrochloride; cocaine hydrochloride; bretylium tosylate; reserpine phosphate (dissolved in 20% ascorbic acid solution).

Working solutions of drugs were made each day from concentrated stock solutions which were kept frozen until used. All working solutions were kept in ice during the experimental period. The concentrations of drugs used (except for $[^3H]$-noradrenaline) are expressed in terms of the salts listed above per ml of medium in the muscle chamber.

Results

Effect of cocaine on the ability of bretylium pretreatment to increase the restoration of responses to tyramine in reserpine-treated atria

The atria were initially tested with tyramine (10 μg/ml for 5 min) and the absence of inotropic response ensured that the degree of reserpinization

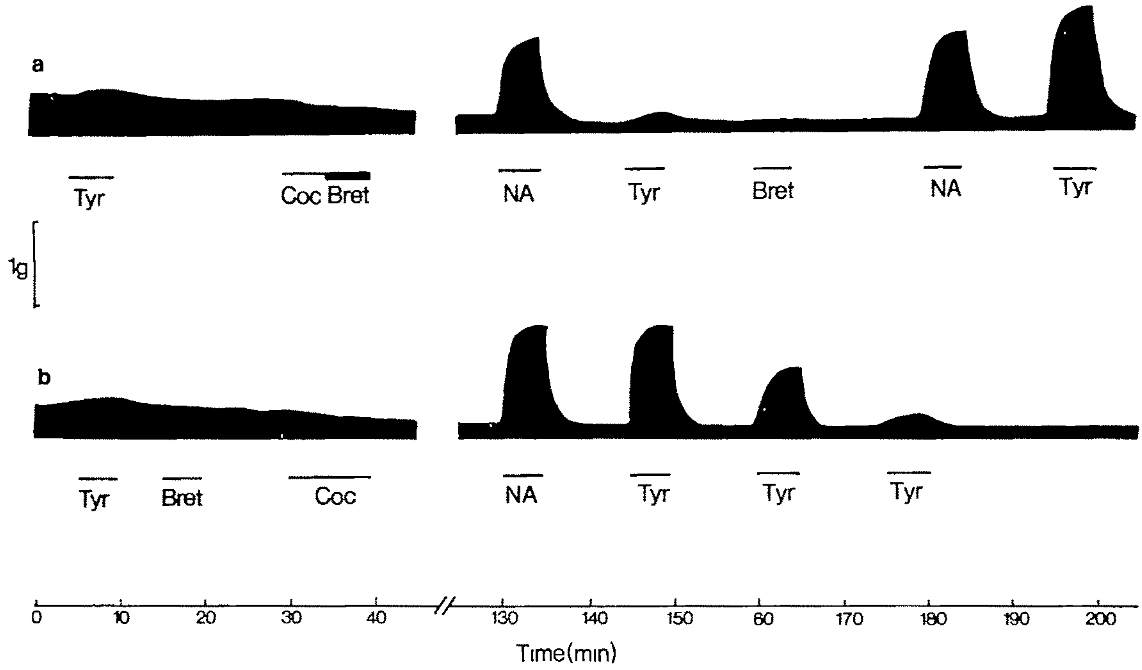

Figure 1 Effect of cocaine (Coc) on the ability of bretylium (Bret) pretreatment to enhance the restoration of the responses to tyramine (Tyr), after incubation with noradrenaline (NA), in reserpine-treated left atrium. Upper (a) and lower (b) records are from two halves of a single left atrium. Concentration of drugs used: tyramine, 10 μg/ml; cocaine 50 μg/ml; bretylium, 20 μg/ml; noradrenaline, 0.5 μg/ml. The records shown were constructed from measurements taken on the original polygraph records.

was adequate. Then the control half (Figure 1b) was exposed to bretylium (20 μg/ml for 5 min) and 15 min later both control and experimental preparations were treated with cocaine (50 μg/ml for 10 minutes). Five min after addition of cocaine (without previous washout) the experimental preparation (Figure 1a) was exposed to bretylium (20 μg/ml for 5 minutes). After washout of cocaine both halves were washed every 15 min for a 90 min period. This precaution was taken in order to avoid the residual effect of cocaine on the uptake mechanism. At the end of this period both preparations were incubated with noradrenaline (0.5 μg/ml for 5 min) and tested with tyramine 15 min later. Figure 1 shows that the tyramine response was completely restored in the control preparation (b), while it was almost absent in the experimental one (a). After subsequent additions of tyramine to the control preparation the inotropic response disappeared. When the experimental atrium was exposed again to bretylium in the absence of cocaine and incubated with noradrenaline, the response to a subsequent addition of tyramine was restored. Similar results were obtained in eight further experiments. It should be noted that in five experiments in which the atrium had been tested for sensitivity to noradrenaline before and at various intervals after cocaine washout, the sensitizing effect of cocaine had practically disappeared 90 min after the first washout.

In six additional experiments, after failing to respond to tyramine, both atrial preparations were treated with cocaine (50 μg/ml for 10 min) and 5 min later, in the presence of cocaine, were exposed to bretylium (20 μg/ml for 5 minutes). After a lapse of 90 min, as in the above group of experiments, one preparation (Figure 2a) was incubated with noradrenaline (0.5 μg/ml for 5 min) while the other (Figure 2b) was incubated with α-methyl-noradrenaline (0.5 μg/ml for 5 min), which is not a substrate for monoamine oxidase. Fifteen min later tyramine (10 μg/ml for 5 min) was added to both halves. Figure 2 illustrates the results of a typical experiment. The tyramine response was restored when α-methylnoradrenaline but not when noradrenaline was used for incubation.

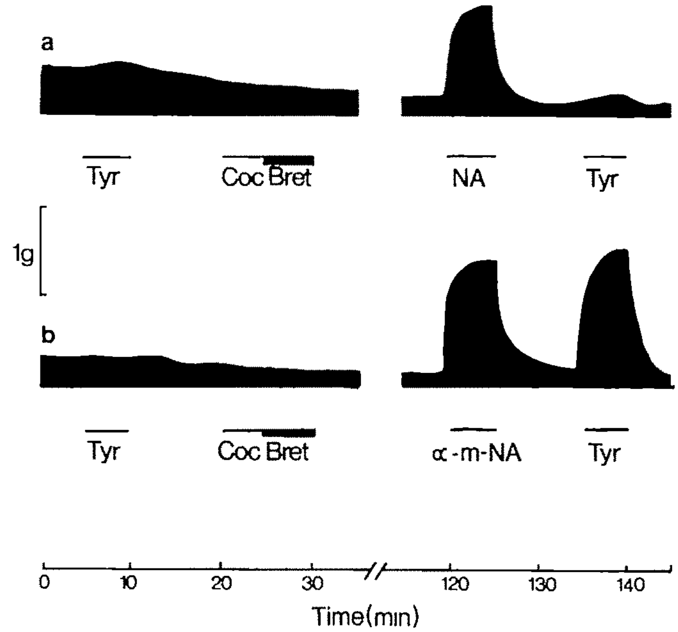

Figure 2 Comparative effectiveness of noradrenaline (NA) and α-methyl-noradrenaline (α-m-NA) in restoring the responses to tyramine (Tyr) in reserpinized left atrium pretreated with bretylium (Bret) in the presence of cocaine (Coc). Upper (a) and lower (b) records are from two halves of a single left atrium. Concentration of drugs used tyramine, 10 μg/ml; cocaine, 50 μg/ml; bretylium, 20 μg/ml; noradrenaline, 0.5 μg/ml; α-methyl-noradrenaline, 0.5 μg/ml. Records were constructed as indicated in Figure 1.

Effect of cocaine on the ability of bretylium pretreatment to increase the uptake-retention of [^{3}H]-noradrenaline by reserpine-treated atria

If cocaine prevents the ability of bretylium pretreatment to restore the inotropic responses to tyramine in reserpinized atria after incubation with noradrenaline, then cocaine treatment should also prevent the increase in accumulation of [^{3}H]-noradrenaline due to bretylium administration. In order to test this possibility a group of experiments were carried out in which a half atrium was treated with bretylium (20 μg/ml for 5 min) and then, after washout, with cocaine (50 μg/ml for 10 min), while the other half was first exposed to cocaine for 5 min and later, with cocaine still present, bretylium was added. After washout of the drugs for a period of 90 min to ensure that the effect of cocaine on the uptake mechanism disappeared, [^{3}H]-noradrenaline (10 μCi) was added to the bath for 5 min, followed by a 45 min washout period. Figure 3 shows that reserpinized tissues which were not treated with bretylium retained very little [^{3}H]-noradrenaline (0.81 ± 0.04 d min^{-1} g^{-1} x 10^{-4}, column A); however, pretreatment with bretylium alone greatly increased uptake-retention of the amine by 20-fold (15.05 ± 1.83 d min^{-1} g^{-1} x 10^{-4}, column B). When cocaine was given after bretylium no change in [^{3}H]-noradrenaline retention was observed as compared to the atrium treated with bretylium alone; however, if cocaine was administered first, and then, still in the presence of the drug, bretylium was given, uptake-retention of [^{3}H]-noradrenaline was significantly impaired (7.20 ± 2.14 as compared to 17.26 ± 4.38 d min^{-1} g^{-1} x 10^{-4} for the control half atrium which received bretylium before cocaine, $P < 0.01$).

Effect of sodium on the ability of bretylium pretreatment to increase the retention of [^{3}H]-noradrenaline in reserpine-treated atria

In this group of experiments both control and experimental halves were kept from the beginning, in a low-sodium medium (25 mM) or in a sodium-free medium (tonicity maintained with sucrose) for a 30 min period. Under these conditions the atria did not beat. Then the experimental preparations were given bretylium (20 μg/ml for 5 min) and then washed with low-sodium or sodium-free

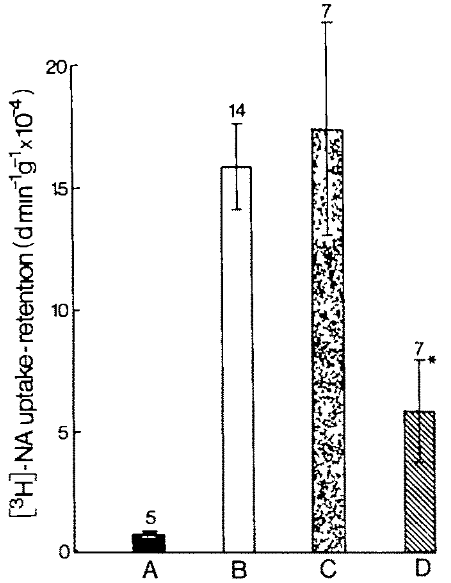

Figure 3 Influence of cocaine on the ability of bretylium to increase the retention of $[^3H]$-noradrenaline ($[^3H]$-NA) by reserpine-treated atria (A) Non-treated atria (B) Atria treated with bretylium (20 µg/ml) prior to incubation with $[^3H]$-noradrenaline. (C) Atria incubated with bretylium, followed by cocaine (50 µg/ml. (D) Atria treated with cocaine and then, in the presence of this drug, with bretylium. Data of columns (C) and (D) are obtained from halves of the same atria. Figures above columns denote the number of experiments. Vertical lines show s.e. mean * $P < 0.01$ (paired comparison, (C) against (D)).

Krebs solution, and 10 and 20 min later with normal Krebs. Ten min after the last washout the control preparation was exposed to bretylium (20 µg/ml for 5 minutes). Twenty min after the washout of the drug both halves were incubated with $[^3H]$-noradrenaline (10 µCi for 5 min), and finally removed from the muscle chamber for analysis 45 min after the first washout. The results are summarized in Figure 4. It can be seen that the mean concentration of $[^3H]$-noradrenaline in the preparation treated with bretylium in 25 mM sodium was only 50% of that found in the corresponding control (6.65 ± 1.91 and 13.31 ± 1.49 d min^{-1} g^{-1} x 10^{-4}, respectively, $P < 0.05$). Further decrease in amine retention was observed in preparations treated with bretylium in the total absence of sodium (3.18 ± 0.65 d min^{-1} g^{-1} x 10^{-4}, $P < 0.01$ when compared with controls). It should be noted that since control preparations were exposed for the same time to low or sodium-free medium, any residual effect of sodium-free exposure on the $[^3H]$-noradrenaline uptake system can be discounted. Lesser retention of $[^3H]$-noradrenaline in control atria in these experiments, as compared with previous controls (Figure 3, column B) may be due to some residual effect of the low-sodium medium on the noradrenaline uptake system.

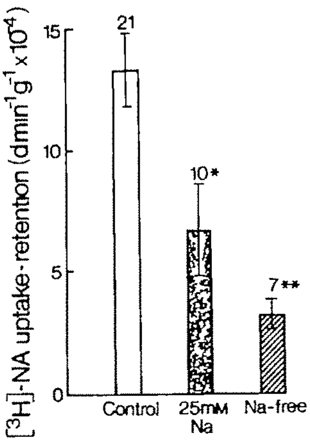

Figure 4 Effect of sodium on the ability of bretylium (20 µg/ml) to increase the retention of $[^3H]$-noradrenaline ($[^3H]$-NA) by reserpine-treated atria. Control preparations were exposed to 25 mM sodium or to sodium-free solutions for the same length of time as experimental preparations (see text) Above columns, number of experiments. Vertical lines show s.e. mean * $P < 0.05$; ** $P < 0.01$ (group comparison).

Effect of potassium deprivation on the ability of bretylium to increase the retention of $[^3H]$-noradrenaline by reserpine-treated atria

In a group of six experiments both halves were kept for 30 min in a medium containing no potassium. One half was then treated with bretylium (20 µg/ml for 5 minutes). Both were then washed with potassium-free Krebs and 10 min later with normal Krebs. Ten min after the last washout the control preparation was exposed to bretylium (20 µg/ml for 5 minutes). Twenty min after washout of the drug both halves were incubated with $[^3H]$-noradrenaline (10 µCi for 5 min), and finally washed for 45 minutes. Uptake-retention of $[^3H]$-noradrenaline in control and experimental preparations was not significantly different (15.72 ± 3.88 d min^{-1} g^{-1} x 10^{-4} in preparations treated with bretylium in normal Krebs as against 15.07 ± 2.58 d min^{-1} g^{-1} x 10^{-4} in atria treated with bretylium in the absence of potassium).

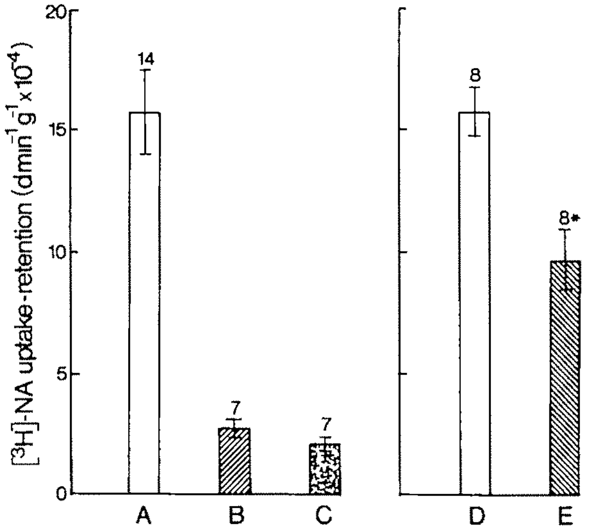

Figure 5 Ability of sodium-free and high-calcium solutions to release bretylium from reserpinized atria. (A) Controls, bretylium (20 μg/ml) was given in normal Krebs solution. (B) Bretylium administered in sodium-free (sucrose). solution. (C) Preparations treated with bretylium in normal Krebs solution followed by 40 min exposure to sodium-free medium. (D) Controls, atria treated with bretylium in normal Krebs. (E) Preparations treated with bretylium in normal Krebs followed by 40 min exposure to 50 mM calcium Krebs. Above columns, number of experiments. Vertical lines show s.e. mean $^* P < 0.01$ compared with (D) (paired comparison).

Effect of sodium deprivation and high calcium on the increase in [^{3}H]-noradrenaline retention after bretylium pretreatment in reserpinized guinea-pig atria

Several investigators have shown that removal of sodium from the incubation medium causes release of noradrenaline from sympathetic nerve terminals (Kirpekar & Wakade, 1968a; Bogdanski & Brodie, 1969; García & Kirpekar, 1973). Therefore, it was of interest to study if exposure to sodium-free medium releases bretylium from atria previously treated with this agent. In a group of 7 paired experiments one half atrium was treated with bretylium (20 μg/ml for 5 min) in sodium-free (sucrose) medium. The atrium was subsequently washed with the same solution and 20 min later with normal Krebs solution. The other half-atrium was incubated with bretylium (20 μg/ml for 5 min) in normal Krebs and then washed twice for 40 min with sodium-free (sucrose) medium. Twenty min after changing to normal Krebs solution both strips were incubated with [^{3}H]-noradrenaline (10 μCi, for 5 min) and washed out in the usual manner. When the initial incubation with bretylium was carried out in a sodium-free (sucrose) medium [^{3}H]-noradrenaline retention was greatly impaired (Figure 5, column B). On the other hand, in the half atrium treated with bretylium in normal Krebs, and then switched to sodium-free (sucrose) Krebs, retention of [^{3}H]-noradrenaline was also markedly decreased (Figure 5, column C). This retention, which amounted to only 13% of controls (column A), was comparable to that in the preparation initially treated with bretylium in the absence of sodium (column B).

Burn & Welsh (1967) showed that bretylium blocked the inhibition of the movements of the rabbit ileum induced by stimulation of the periarterial nerves, when the calcium concentration was raised the bretylium block was removed. In order to test the possibility that high calcium could also reverse the enhanced retention of [^{3}H]-noradrenaline induced by bretylium, a group of 8 experiments was carried out in which bretylium (20 μg/ml, for 5 min) was administered in normal Krebs to both halves of a reserpinized atrium; the medium of one preparation was then changed to Krebs containing calcium 50 mM for 40 min and then back to normal Krebs 20 min before [^{3}H]-noradrenaline (10 μCi for 5 min) incubation started. Figure 5 (column E) shows that [^{3}H]-noradrenaline uptake-retention by the tissue treated with high calcium solution was significantly decreased by 42% ($P < 0.05$) as compared to controls (column D).

Discussion

Atria from guinea-pigs whose noradrenaline stores have been depleted by pretreatment with reserpine are essentially unresponsive to tyramine. The response to tyramine is not appreciably increased if the atria are then incubated with noradrenaline alone, but is more strongly restored if the atria are pretreated with monoamine oxidase inhibitors or bretylium (Furchgott *et al.*, 1963; Furchgott & Sánchez-García, 1966, 1968; Furchgott *et al*, 1971). It thus appears that bretylium behaves like a classical monoamine oxidase inhibitor as far as restoration of tyramine response to noradrenaline is concerned.

In the present study it was found that cocaine, when present during bretylium treatment, prevents bretylium from enhancing the effect of a brief incubation with noradrenaline in the restoration of the inotropic response to tyramine in reserpinized guinea-pig left atrium. The possibility that a residual effect of cocaine, on the uptake mechanism for noradrenaline could account for this phenomenon is excluded by the following considerations: (1) the residual sensitization to noradrenaline had disappeared 90 min after washout of cocaine, which was exactly the length of the interval between cocaine treatment and the incubation with noradrenaline; (2) when the atrium was exposed to bretylium before the addition of cocaine, the inotropic response to tyramine was restored in a normal manner which indicates that uptake of noradrenaline was normal; (3) when bretylium was added in the presence of cocaine, which, as has been shown, prevents the restoration of the response to tyramine when the preparation was incubated with noradrenaline, a subsequent incubation with α-methylnoradrenaline still restored the response to tyramine (both noradrenaline and α-methyl-noradrenaline are about equal in potency as inotropic agents on the guinea-pig left atrium; Furchgott & Sánchez-García, 1968).

Our results can be satisfactorily explained on the hypothesis that cocaine and bretylium compete for the uptake sites in adrenergic nerve terminals. Restoration of the inotropic response to tyramine in reserpinized atria by bretylium was markedly inhibited if atria were exposed to bretylium in the presence of cocaine. This result suggests that cocaine prevented bretylium from accumulating within the adrenergic nerve terminals. Since monoamine oxidase inhibition by bretylium appears to be related to the intraneuronal concentration of this agent (Furchgott, 1964; Furchgott & Sánchez-García, 1966; Furchgott *et al.*, 1971), the noradrenaline taken up during incubation would be rapidly deaminated, resulting in failure to restore the response to tyramine. Moreover, the interpretation is supported by the observation that under the same conditions the restoration of the response to tyramine took place normally when α-methylnoradrenaline was used for incubation. Our results are in agreement with previous findings of Kirpekar & Furchgott (1964) who showed that cocaine antagonizes the inotropic effect of bretylium on normal guinea-pig left atrium.

Brodie, Chang & Costa (1965) have shown that bretylium uptake by rat heart *in vivo* decreases by about 32% in animals previously treated with cocaine. Although our results on restoration of tyramine response do not allow us to measure exactly the degree of blockade of bretylium uptake, the fact that cocaine when present during bretylium treatment completely abolished the restoration of the response to tyramine, suggests that blockade must be appreciable. Studies on $[^3H]$-noradrenaline uptake-retention after bretylium treatment allow us to measure more accurately the degree of blockade of bretylium uptake by cocaine. It has been shown that bretylium increases the retention of $[^3H]$-noradrenaline in reserpinized atria (Furchgott *et al.*, 1971) and this property of bretylium has been used here as an index of bretylium uptake. Experiments summarized in Figure 3 indicate that the degree of blockade of bretylium uptake into adrenergic nerve terminals by cocaine was at least of the order of 70%. The small amount of bretylium taken up into the nerves in the presence of cocaine was probably not sufficient to permit restoration of the response to tyramine by a previous incubation with noradrenaline.

Noradrenaline uptake is dependent on the presence of sodium ions (Iversen & Kravitz, 1966; Gillis & Paton, 1967; Kirpekar & Wakade, 1968b; Horst, Kopin & Ramey, 1968; Bogdanski & Brodie, 1969). The results of the present study show that sodium is also required for the uptake of bretylium into the adrenergic nerve terminals of the guinea-pig atrium. When tissues were pretreated with bretylium in a sodium-deficient or sodium-free medium, the retention of $[^3H]$-noradrenaline was only 50% and 25%, respectively, of that found in the corresponding control atrium. A diminished intraneuronal monoamine oxidase inhibition, and therefore a more rapid deamination of the noradrenaline taken up as a consequence of the blockade of bretylium uptake into adrenergic nerve endings, provides an explanation of the results. The possibility that this phenomenon could be due to a residual effect of the sodium-deficient medium on noradrenaline uptake is excluded if one takes into account that both the control and the experimental preparation were kept in such a medium for the same length of time.

Even though the absolute requirement of sodium for the membrane uptake system for noradrenaline is clearly established (Iversen & Kravitz, 1966; Bogdanski & Brodie, 1966; Gillis & Paton, 1967), there are contradictory reports concerning the role of potassium on the uptake of noradrenaline into the sympathetic nerve terminals. Gillis & Paton (1967) showed a marked reduction in the retention of noradrenaline by heart slices in the absence of potassium but, Kirpekar & Wakade (1968b) could not demonstrate any role of potassium ions on the inactivation of noradrenaline infused in the cat spleen. We also showed that potassium did not affect the accumulation of bretylium into the sympathetic nerve terminals and therefore the increased retention of [^{3}H]-noradrenaline.

Toda (1972) found that the inhibition of responses of atria and aortae to transmural stimulation by bretylium was prevented by cocaine, but not by sodium deficiency. The author concluded that antagonism of bretylium effects by cocaine is not related to the property of cocaine of inhibiting the noradrenaline transport system. However, at the sodium concentration used by the author (64% of normal), the uptake of noradrenaline is reduced by only 20% (Iversen & Kravitz, 1966). In contrast, in our experiments, a low-sodium medium (25 mM) or complete removal of sodium drastically decreased the enhanced retention of noradrenaline induced by bretylium, indicating a blockade of bretylium uptake into the nerve terminals probably through the same membrane uptake system as that for noradrenaline.

There is ample evidence that sodium is not only necessary for the uptake of noradrenaline, but that it is required also for the storage of the amine in the sympathetic nerve terminals. Tissues lose their noradrenaline content when exposed to a low-sodium environment (Kirpekar & Wakade, 1968a; Bogdanski & Brodie, 1969; García & Kirpekar, 1973). Experiments carried out to see if bretylium was released from the sympathetic nerve terminals by exposure to sodium-free medium clearly demonstrated that like noradrenaline, bretylium was released leading to an impairment of the ability of the tissue to retain [^{3}H]-noradrenaline.

Bretylium seems to be released from the nerve terminals by exposure of the atria to a high calcium solution (50 mM). These results agree well with those of Burn & Welsh (1967), who showed that stimulation of the periarterial nerves inhibits the movements of the rabbit ileum, and that bretylium and guanethidine block this effect; the inhibition of the movements by nerve stimulation was restored by raising the extracellular calcium concentration. Kirpekar, Wakade, Dixon & Prat (1969) suggested that calcium competes with guanethidine for the site in the nerve endings at which the latter acts to inhibit noradrenaline release. Our results also suggest that calcium may compete with and displace bretylium from the specific intracellular sites on which the drug produces its inhibitory action on monoamine oxidase.

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DISCRIMINATION OF MONOAMINE UPTAKE BY MEMBRANES OF ADRENAL CHROMAFFIN GRANULES

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1 The accumulation of various radioactive monoamines by isolated membranes of bovine adrenal chromaffin granules was measured by equilibrium dialysis.

2 Adenosine-5′-triphosphate (ATP) in the presence of Mg^{++} stimulated the uptake of all the amines tested, but the accumulation of dopamine, (−)-noradrenaline (NA), 5-hydroxytryptamine (5-HT), (±)-adrenaline and (±)-octopamine was greater than that of tyramine, (±)-metaraminol, tryptamine, β-phenylethylamine and histamine.

3 At the higher concentration levels of the amines in the medium the ATP-dependent accumulation of dopamine, NA, adrenaline and 5-HT in the membranes reached a saturation level, whereas in the absence of the nucleotide no saturation level was attained.

4 Octopamine and 5-HT competitively inhibited the ATP-dependent uptake of NA.

5 Decrease in the incubation temperature or the presence of N-ethylmaleimide greatly reduced the ATP-stimulated amine accumulation. Ouabain had no effect on uptake.

6 Reserpine virtually abolished the ATP-dependent uptake of dopamine, NA and 5-HT, caused a partial inhibition of the metaraminol, octopamine and tyramine accumulation, but did not interfere with the uptake of tryptamine.

7 The content of endogenous catecholamines of the membranes was changed very little by incubation of NA and 5-HT in the presence of ATP. However, the membranes lost over 80% of their endogenous amines if incubated for 30 min without ATP.

8 The ATP content of the medium progressively decreased during the incubation of granular membranes.

9 It is concluded that the membrane of adrenal chromaffin granules discriminates between the various monoamines with regard to the magnitude of their uptake and that two mechanisms of ATP-stimulated uptake, one responsive and the other resistant to reserpine, exist at the level of this membrane. The ATP-stimulated transport at the granular membrane level may be an important factor in determining the intraneuronal storage of a physiological or false neurotransmitter.

Introduction

The amine storage organelles, e.g. of noradrenergic and 5-hydroxytryptaminergic neurones, are able to take up and accumulate amines other than those which they contain under physiological conditions. For instance, in the central nervous system L-DOPA causes an accumulation of dopamine not only in dopaminergic but also in 5-hydroxytryptaminergic neurones (Bartholini, Da Prada & Pletscher, 1968, Ng, Chase, Colburn & Kopin, 1970), and α-methyl-dopa leads to an increase of α-methyl-dopamine and α-methyl-noradrenaline in catecholaminergic neurones (Muscholl, 1972). Experiments with intact isolated amine storage granules, e.g. from blood platelets (Da Prada & Pletscher, 1968) or adrenal medulla (Carlsson, Hillarp & Waldeck, 1963) showed that the various monoamines are not taken up to the same extent. This may be due to differences in the aggregation of the amines with nucleotides (especially adenosine-5′-triphosphate, ATP) and bivalent cations present within the organelles. In fact, such differences have been shown to exist in artificial solutions (Berneis, Da Prada & Pletscher, 1969). However, it is not known whether in addition the granular membrane has the ability to discriminate between the various amines with regard to their uptake.

In the present work the uptake of different monoamines by isolated membranes of bovine adrenal chromaffin granules is compared by a new method of equilibrium dialysis.

Methods

Preparation of membranes

Chromaffin granules were isolated from bovine adrenal glands (Smith & Winkler, 1967) with a post mortem delay of about 30 minutes. The isolated granules, obtained from 5-8 g medullary tissue, were lysed by hypotonic shock in 10 ml 0.015 M KCl and their membranes isolated by centrifugation at 39,000 x *g* for 10 minutes. After one washing of the sedimented membranes in 10 ml hypotonic KCl, they were resuspended in 7 ml 0.15 M KCl and dialysed for 20 min against 4 litres 0.15 M KCl using a b/HFD 1/20 Mini beaker dialyzer with cellulose hollow fibres (Bio-Rad Labs, Richmond, Cal., USA). Subsequently, the membranes were again sedimented by centrifugation and washed twice with 10 ml 0.15 M KCl. After suspending the membranes for the second washing the protein content was roughly estimated by measuring the light absorption of the suspension at 280 nm. A portion of the membranes was analysed for endogenous ATP and catecholamines. The remaining membranes were resuspended in 4-8 ml of 50 mM Na-glycerophosphate buffer, pH 7.4, containing 0.1 mM ascorbic acid, 5 mM MgCl$_2$ and 10 µM pargyline (*N*-benzyl-*N*-methyl-propargylamine, an inhibitor of monoamine oxidase; Pletscher, Gey & Burkard, 1966) (Taugner, 1971) so as to obtain a final concentration of about 800 µg protein per ml. In some experiments the buffer was supplemented with 5 mM ATP. All procedures were performed at 4°C.

Equilibrium dialysis

A 'Dianorm' equilibrium dialyzer (Diachema AG, Birmensdorf/Zürich, Switzerland), made of Teflon and containing two microchambers, separated by a 'spectrapor 2 T M membrane' (mol. wt cutoff: 12,000-14,000) (Spectrum Medical Industries, Inc., Los Angeles) (Figure 1) was used for all experiments. An aliquot of 125 µl of the final membrane suspensions was placed in one chamber and the same volume of buffer devoid of membranes in the other. Equal amounts of the radioactive amines, in a volume of 10 µl, were injected into each chamber.

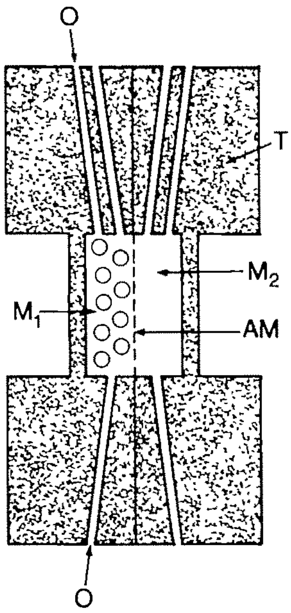

Figure 1 Model of microdialyzer AM = semi-permeable artificial membrane; M$_1$ = microchamber (200 µl) containing biological membranes; M$_2$ = microchamber (200 µl) containing incubation medium only; O = open outlet (plugged during incubation) for filling or removal of incubation mixture; T = Teflon. After filling, five dialyzers were fixed on a rotor and submerged in a water bath in order to maintain a constant temperature.

Equilibrium dialysis experiments were carried out at various temperatures, incubation times and amine concentrations. At the end of an experiment, 40 µl aliquots, in duplicate, were extracted from each chamber, placed in 10 ml of scintillation mixture (Aquasol, New England Nuclear, Boston, USA) and counted in an Isokap/300 liquid scintillation system (Nuclear, Chicago, USA). The difference between the counts in the chamber containing the biological membranes and those in the chamber without membranes represents the amount of the amine incorporated into the membrane preparation. The quantity of the amine taken up by the membranes was expressed in nmol per mg protein. Virtually no radioactive amines were adsorbed onto the artificial membrane and the Teflon walls of the chambers.

Assay methods

Endogenous catecholamines were determined by spectrophotofluorimetry (Laverty & Taylor, 1968). The ATP of the membranes (about 400 µg proteins) was extracted three times with 0.5 ml HClO$_4$, adjusted to pH 6.0 with K$_2$CO$_3$, injected into a Varian Aerograph LCS 1000 ion exchange liquid chromatograph (Varian, California, USA) (Brown, 1973) and measured by absorption at 254 nm. In some experiments this method was also used for determining the content of ATP, adenosine-5′-diphosphate (ADP) and adenosine-5′-monophosphate (AMP) of the medium which was injected into the aerograph after dilution with 0.1 M phosphate buffer, pH 6.0. In other experiments the determination of ATP in the medium was carried out by the luciferin-luciferase method (Holmsen, Holmsen & Bernhardsen, 1966). Proteins were estimated colorimetrically (Lowry, Rosebrough, Farr & Randall, 1951).

Materials

Adenosine-5′-triphosphate disodium salt, Sigma grade, was obtained from Sigma, St Louis, Mo., USA; (±)-adrenaline-[carbinol-^{14}C] (±)-bitartrate (50 mCi/mmol), 3,4-dihydroxyphenyl[ethylamine-2-^{14}C] hydrochloride (55 mCi/mmol), 5-hydroxytryptamine-[3′-^{14}C]-creatinine sulphate (55 mCi/mmol), histamine-[ring-2-^{14}C] dihydrochloride (54 mCi/mmol), (−)-noradrenaline-[carbinol-^{14}C] (±)-bitartrate (37 mCi/mmol) and tyramine-[1-^{14}C] hydrochloride (43.7 mCi/mmol) from the Radiochemical Centre, Amersham, Bucks., (±)-metaraminol-[7-^{3}H] (N) (5 Ci/mmol), (±)-octopamine-[2-^{3}H] (N) (1 Ci/mmol), tryptamine-[2-^{14}C]-bisuccinate (10 mCi/mmol) and β-phenylethylamine-[1-^{14}C] hydrochloride (7 mCi/mmol) from New England Nuclear, Boston, USA. All other substances used were of analytical grade. The purity of the radioactive compounds indicated by the suppliers was confirmed by paper chromatography.

Results

Noradrenaline

The accumulation of (−)-noradrenaline (NA) in the isolated membranes of chromaffin granules was greatly stimulated by the presence of ATP in the incubation medium. The uptake increased with the time of incubation and reached a maximum between 30 and 60 minutes. Thereafter, the amounts of labelled NA in the membranes decreased, but were still elevated at 150 min (Figure 2). In the absence of ATP, the amount of NA which accumulated was greatly reduced (Table 1), and no maximum occurred at 30-60 minutes.

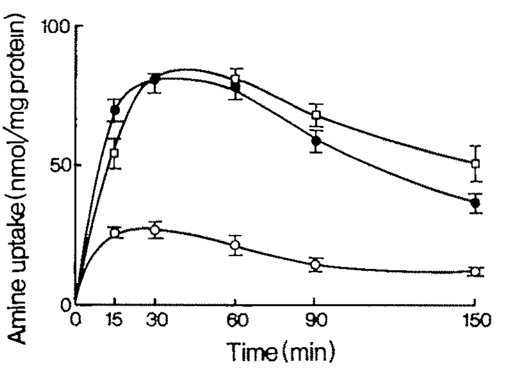

Figure 2 Time course of the uptake of labelled (−)-noradrenaline (□), dopamine (●) and tryptamine (○) by isolated membranes of bovine adrenal chromaffin granules incubated at 37°C in MgCl$_2$-containing medium in the presence of adenosine-5′-triphosphate (ATP). Initial concentrations: amines 45 μM, ATP 5 mM The points are mean of 3-8 experiments. Vertical bars show s.e mean

The uptake of NA during the first 30 min also rose with increasing concentration of the radioactive amine. In the presence of ATP, a saturation level was reached at amine concentrations of 200-400 μM. Without ATP, the NA showed much less accumulation in the membrane over the whole concentration range (10-600 μM). The saturation level of NA in the presence of ATP was about 100 times higher than the corresponding amine levels obtained in the absence of the nucleotide (Figure 3).

Other amines

The accumulation by membranes of adrenal chromaffin granules of all the other amines tested was stimulated by ATP. The magnitude of the ATP-stimulated uptake, however, showed considerable differences (Table 1). For instance, dopamine, NA, 5-HT, (±)-adrenaline and (±)-octopamine were taken up to a greater extent than tyramine, tryptamine, (±)-metaraminol, phenylethylamine and histamine. The accumulation of dopamine and histamine differed by a factor of about 15.

The uptake of dopamine and tryptamine in the presence of ATP followed a time course similar to that of NA, i.e. it reached a maximum after 30-60 min with a subsequent drop (Figure 2). Other amines, e.g. 5-HT, behaved similarly. The small uptake which occurred in the absence of ATP did not exhibit this maximum. Dopamine, 5-HT and adrenaline also showed a concentration-dependence similar to NA. Thus, in the presence of ATP a saturation level was attained at 200-400 μM, whereas in the absence of ATP a

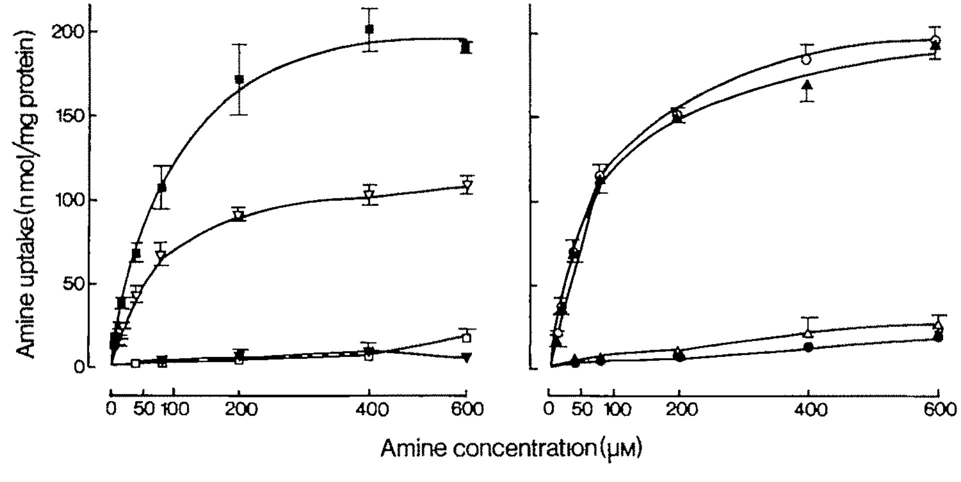

Figure 3 Uptake of labelled (—)-noradrenaline (NA), (±)-adrenaline (Ad), dopamine (DA) and 5-hydroxytryptamine (5-HT) by isolated membranes of bovine adrenal chromaffin granules incubated at 37°C for 30 min in $MgCl_2$-containing medium with various concentrations of the amines in the presence and absence of adenosine-5'-triphosphate (ATP, initial concentration 5 mM). The points are mean of 3-4 experiments. Vertical bars show s.e mean. (■) NA + ATP; (□) NA, (▼) Ad; (▽) Ad + ATP, (▲) 5-HT + ATP, (△) 5-HT; (●) DA; (○) DA + ATP.

much less marked uptake occurred over the whole concentration range (Figure 3).

Both octopamine (Figure 4) and 5-HT inhibited the uptake of NA by adrenal granular membranes. According to the double reciprocal plots, i.e. $1/V$ versus $1/(S)$ (Figure 4) and V versus $V/(S)$, the inhibition was of the competitive type. The K_m for NA amounted to 11×10^{-5}, the K_i for octopamine and 5-HT to 17×10^{-5} and 3.5×10^{-5}, respectively.

Interference with ATP-stimulated uptake

The ATP-stimulated uptake of all the monoamines tested decreased considerably when the temperature of the incubation medium was diminished,

Table 1 Uptake of various radioactive amines by isolated membranes of bovine adrenal chromaffin granules incubated in a $MgCl_2$-containing medium at 37°C for 30 minutes

Incubation with	*Presence of ATP*	*Absence of ATP*
Dopamine	80.09 ± 7.26	2.49 ± 0.32
(—)-Noradrenaline	76.73 ± 5.56	4.30 ± 0.86
5-Hydroxytryptamine	74.04 ± 7.20	4.02 ± 1.23
(±)-Adrenaline	64.64 ± 6.77	2.21 ± 0.57
(±)-Octopamine	54.02 ± 3.39	1.38 ± 0.47
Tyramine	37.93 ± 3.08	1.62 ± 0.03
(±)-Metaraminol	28.86 ± 0.93	0.65 ± 0.27
Tryptamine	22.47 ± 0.77	10.52 ± 1.23
Phenylethylamine	14.28 ± 0.85	2.20 ± 0.58
Histamine	5.32 ± 0.40	1.68 ± 0.44

Initial concentrations in incubation medium: amines 45 μM, adenosine-5'-triphosphate (ATP) 5 mM. The figures represent mean with s.e. mean of 3-6 experiments and are indicated in nmol per mg membrane protein.

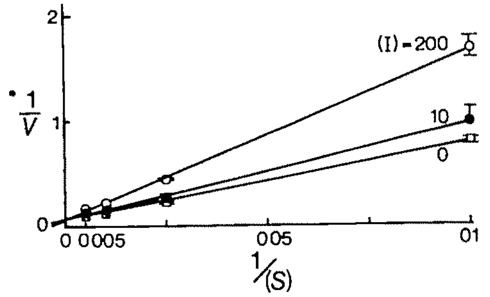

Figure 4 Double reciprocal plot (Lineweaver-Burk) of the effect of octopamine on the uptake of (−)-[^{14}C]-noradrenaline (NA) by membranes of adrenal chromaffin granules incubated for 10 min at 37° C in MgCl$_2$-containing medium in the presence of ATP (initial concentration 5 mM) V = initial velocity of uptake (nmol per mg of protein per 10 min), S = molar concentration of NA (I) = concentration of octopamine in μM. Each value is the mean of 4-8 experiments. Vertical bars show s.e. mean.

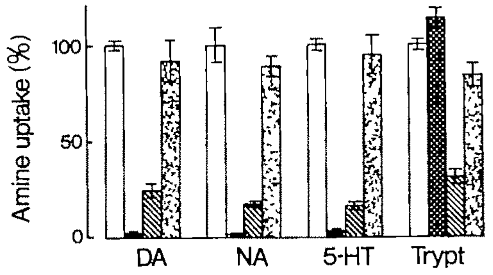

Figure 5 Effect of reserpine (10^{-5} M; cross-hatched), N-ethylmaleimide (diagonally hatched; 5 x 10^{-4} M) and ouabain (5 x 10^{-4} M, stippled) on the uptake of labelled dopamine (DA), (−)-noradrenaline (NA), 5-hydroxytryptamine (5-HT) and tryptamine (Trypt) by isolated membranes of bovine adrenal chromaffin granules incubated for 30 min at 37° C in MgCl$_2$-containing medium in the presence of ATP (initial concentration 5 mM) Open columns, controls The columns are mean of 3-5 experiments expressed as percentage of the values obtained in controls, not incubated with inhibitors (=100%). Vertical bars show s e. mean

the accumulation in the membranes at 2° C approximately corresponding to that seen at 37° C in the absence of ATP (Table 2)

N-ethylmaleimide, but not ouabain, greatly inhibited the ATP-dependent uptake of dopamine, 5-HT, NA and tryptamine (Figure 5).

The action of reserpine on the ATP-stimulated uptake differed for the various amines. The drug caused a dose-dependent inhibition of the accumulation of NA, octopamine and metaraminol. However, the inhibition of the uptake of NA was considerably greater than that of metaraminol and slightly more marked than that of octopamine (Figure 6). Dopamine and 5-HT behaved like NA (Figure 5) and tyramine like metaraminol. In contrast, reserpine in concentrations as high as 10^{-5} M did not decrease the uptake of tryptamine (Figure 5).

Endogenous catecholamines and ATP

In three experiments, the content of endogenous catecholamines in the washed membranes

Table 2 Temperature dependence of uptake of labelled amines by isolated membranes of bovine adrenal chromaffin granules incubated in a MgCl$_2$-containing medium for 30 minutes

	Incubation temperature, °C		
Incubation with	*2*	*25*	*37*
Dopamine	2.7 ± 1.3	50.9 ± 8.4	80.09 ± 7.26
(−)-Noradrenaline	0.8 ± 0.2	34.1 ± 6.4	76.73 ± 5.56
5-Hydroxytryptamine	3.4 ± 0.9	56.1 ± 8.6	74.04 ± 7.20
(±)-Adrenaline	0.8 ± 0.2	25.0 ± 7.7	64.64 ± 6.77
(±)-Octopamine	1.2 ± 0.2	19.6 ± 4.4	54.02 ± 3.39
Tyramine	1.2 ± 0.2	34.6 ± 3.1	37.93 ± 3.08
(±)-Metaraminol	0.3 ± 0.3	9.2 ± 2.9	28.86 ± 0.93
Tryptamine	4.0 ± 0.0	19.2 ± 0.3	22.47 ± 0.77

Initial concentrations in incubation medium: amines 45 μM, adenosine-5'-triphosphate 5 mM. The values represent mean with s.e. mean of 3 experiments and are indicated in nmol per mg membrane protein

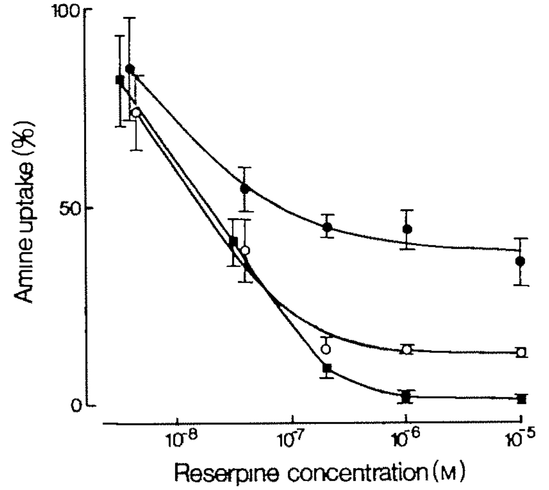

Figure 6 Effect of various concentrations of reserpine on the uptake of labelled (−)-noradrenaline (■), (±)-octopamine (○) and (±)-metaraminol (●) at 37° C by isolated chromaffin granular membranes incubated for 30 min at 37° C in $MgCl_2$-containing medium in the presence of ATP. Initial concentrations: labelled amines 45 μM, ATP 5 mM. The points are mean of 4-6 experiments and are expressed as a percentage of the values obtained after incubation of membranes in the same media but without reserpine (controls, uptake = 100%) The uptake of NA, octopamine and metaraminol in the absence of ATP amounted to 4 ± 1%, 2 ± 2% and 1 ± 1% respectively compared to the controls. Vertical bars show s.e. mean.

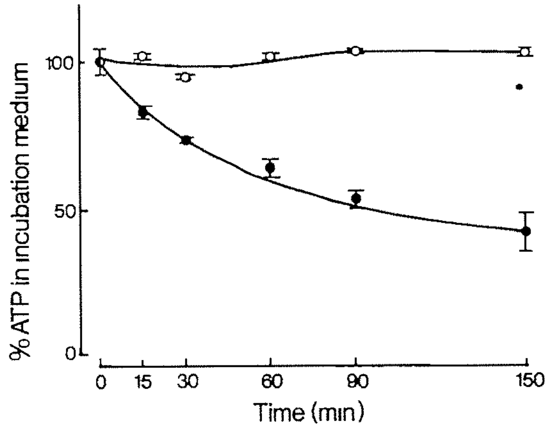

Figure 7 Decrease of the ATP content of the medium during incubation at 37° C with adrenal chromaffin membranes. (○) Without membranes; (●) with membranes Initially the medium contained ATP 5 mM and $MgCl_2$ 5 mM. The points are mean of 5-6 experiments and are indicated as percentages of the 0 values (=100%). Vertical bars show s.e. mean. The measurements of ATP were carried out with the luciferin-luciferase method (see methods section).

amounted to 86.6 ± 8.5 nmol/mg protein. The amine level was not significantly changed ($P > 0.01$) by incubation of the membranes at 37° C for 30 min in the presence of ATP, but decreased to 17.2 ± 1.0% (three experiments) during incubation for 30 min in the absence of ATP. On incubation of membranes for 30 min at 37° C with 45 μM NA or 5-HT in the presence of ATP their endogenous catecholamine content changed very little (Table 3).

The concentration of endogenous ATP in the washed membranes amounted to 4.56 ± 0.27 nmol per mg protein (three experiments).

Degradation of ATP

In suspensions of isolated membranes incubated in the presence of ATP 5 mM the content of this nucleotide decreased with time (Figure 7). After 150 min the incubation medium contained only 2.11 ± 0.32 μmol ATP/ml (initial concentration 5 mM), but 2.23 ± 0.03 μM ADP and 0.36 ± 0 02 mM AMP were also present (three

Table 3 Effect of (−)-[^{14}C]-noradrenaline (NA) and [^{14}C]-5-hydroxytryptamine (5-HT) (initial concentration both 45 μM) on endogenous catecholamines (CA) of chromaffin granular membranes incubated at 37°C for 30 min in a $MgCl_2$-containing medium in the presence of adenosine-5′-triphosphate (5 mM)

	Amines in membrane (nmol/mg protein)			
Incubation with	*0 min*	*30 min*		
	Endogenous CA	*Total CA*	*^{14}C-amines*	*Endogenous CA*
[^{14}C]-NA	105.9 ± 5.4	239.0 ± 14.0	120.1 ± 5.0	118.9 ± 26.0
[^{14}C]-5-HT	105 9 ± 5.4		110.1 ± 6.6	86.0 ± 4.5

In the experiments with NA the amount of endogenous CA after 30 min was calculated by subtracting the labelled amine from the total CA. Each figure indicates mean with s.e. mean of 4 experiments.

experiments). Addition of dopamine 45 μM to the ATP-containing incubation medium had little effect on the decrease of the ATP content.

Discussion

The method of equilibrium dialysis used in the present work for measuring the uptake of biogenic amines by membranes of adrenal chromaffin granules proved to be simpler and more sensitive than previously applied techniques. Thus, the amounts of membranes used (about 100 μg protein per experiment) were smaller than in earlier experiments, and no separation and washing of the membranes had to be carried out at the end of the incubation period. Furthermore, in the presence of ATP the uptake of NA (initial concentration 45 μM) was considerably higher than that seen previously (about 80 versus 10 nmol per mg membrane protein) (Table 1) (Taugner, 1972).

The diminution of the amine uptake which followed the maximum amine accumulation after 30-60 min (Figure 2) might be connected with damage to the membranes caused by prolonged incubation in an artificial medium. This is confirmed by preliminary experiments where it was found that after preincubation at 37°C for 180 min in the presence of ATP 5 mM, the membranes reincubated in fresh medium showed a decrease of their dopamine uptake to 17 ± 4% ($n = 3$) compared to non-preincubated controls (=100%). The amine uptake may also be diminished by decrease of the ATP concentration in the medium in the course of the incubation (Figure 7) and by accumulation of ADP which has been shown to inhibit the ATP-stimulated NA uptake (unpublished results). The measurements of the ATP-dependent amine uptake must therefore be carried out during the initial period of incubation (e.g. after 15-30 minutes).

The uptake results with radioactive NA and adrenaline described above agree with previous findings which strongly suggested the existence in granular membranes of a specific ATP- (and Mg^{++}-) sensitive mechanism besides a less specific and much less efficient process independent of ATP (Taugner, 1972). The present experiments indicate that the content of endogenous catecholamines in adrenal granular membranes also depended on ATP. In fact, incubation of the membranes at 37°C for 30 min in media devoid of ATP decreased the membrane content of endogenous amines to one-sixth (compared to preincubation values), whereas in the presence of ATP little diminution was to be seen.

Several other amines (Table 1) behaved like NA and adrenaline with regard to their accumulation by membranes of chromaffin granules. In fact, in Mg^{++}-containing medium the uptake of these amines (like that of NA and adrenaline) was stimulated by ATP, followed saturation kinetics, was inhibited by a decrease in temperature, by N-ethylmaleimide and in part also by reserpine, whereas ouabain had little effect. Moreover, amines such as octopamine and 5-HT competitively inhibited the ATP-stimulated uptake of NA. These findings indicate that membranes of chromaffin granules also accumulate 'foreign' amines by a specific transport process and that the energy used for this uptake probably derived from the hydrolysis of ATP by the Mg^{++}-dependent ATPase present in the membrane (Hillarp, 1958). The exact mechanism of this transport is not known. It has been suggested that the ATP-ATPase system acts by transphosphorylation of a hypothetical membrane carrier (Slotkin, 1973).

The ATP-stimulated uptake of NA and 5-HT by the granular membranes occurred without a marked concomitant decrease of the endogenous catecholamines. Therefore, the accumulation of the major part of the radioactive amines was not due to an exchange with the endogenous catecholamines, but to a net amine uptake. The site of accumulation of the amines in the membrane preparations in the presence of ATP cannot be established from the results of the present experiments. However, according to previous findings the majority of NA seemed to accumulate in the interior of the membrane vesicles (newly formed from membrane fragments), whereas only minor amounts were bound to the membranes (Agostini & Taugner, 1973).

These findings together with previous results (Da Prada, Berneis & Pletscher, 1971) indicate that ATP has a double function in the accumulation of amines in storage organelles. The nucleotide located within the organelles forms a storage complex by aggregation with the amines, bivalent cations and possibly proteins (chromogranins) (Berneis, Goetz, Da Prada & Pletscher, 1973), whereas another part of ATP is involved in a specific transport of the amines at the level of the granular membranes. It is of interest that there were considerable differences in the ATP-stimulated uptake of the various amines by the granular membranes. Amines like dopamine, NA, 5-HT, whose physiological concentration in certain neurones (e.g. of mammalian brain) is very high, accumulated to a greater extent than others such as histamine, phenylethylamine, tryptamine and tyramine. Therefore, the transport at the granular membrane level might be an important factor in determining the degree of accumulation of a physiologically occurring amine or of a false transmitter in storage organelles of the peripheral sympathetic nervous system and the brain. In this connection it may be of interest that octopamine, whose uptake was stimulated by ATP in the present experiments, accumulated *in vivo* after inhibition of monoamine oxidase and has been claimed to function as a false neurotransmitter (Kopin, Fisher, Musacchio, Horst & Weise, 1965).

The reason why, in the presence of ATP, the granular membrane takes up various amines to a different degree remains to be investigated. The existence of differences in the stereochemical conformation of the amines with regard to that of a hypothetical carrier or receptor is a possibility to be considered.

The above data on the uptake of the amines may in part need some correction Thus, according to preliminary results some dopamine was transformed into NA (about 50%) during the incubation, and a partial transformation of tyramine to octopamine by adrenal granular membranes (which contain dopamine-β-hydroxylase) has also been shown. However, the presence of a β-hydroxy group was probably not a major requirement for uptake since both NA and dopamine accumulated to about the same extent although the latter was only partially β-hydroxylated. Another source of error may result from the comparison of the (−)-form of NA with the racemates of adrenaline, metaraminol and octopamine. Thus, earlier experiments with (−) and (+)-NA (Taugner, 1972) indicated that the uptake of the enantiomers by chromaffin granular membranes may be somewhat different in magnitude.

In previous experiments, reserpine administered *in vivo* has been shown to interfere partially with the granular uptake of metaraminol (Almgren & Lundborg, 1970) The present results clearly demonstrate that reserpine also partially inhibits the metaraminol uptake *in vitro* and that the reserpine-sensitive mechanism is located in the granular membrane On the other hand, reserpine interfered less with the uptake of metaraminol than with that of NA, dopamine and 5-HT. This finding confirms earlier results according to which the granular uptake of metaraminol showed partial reserpine resistance (Lundborg, 1966; Lundborg & Stitzel, 1967; Almgren & Lundborg, 1970; Slotkin & Kirshner, 1971). In addition, the present experiments showed that tyramine and to a small extent octopamine seemed to be partly reserpine-resistant, whereas tryptamine was totally resistant to the alkaloid.

The nature of the reserpine-resistant uptake is not known. However, the present experiments indicate that this uptake is stimulated by ATP. In fact, in media containing relatively high concentrations of reserpine (10^{-5} M) the accumulation of metaraminol, octopamine and tryptamine in granular membranes was enhanced by the nucleotide (compare Figures 5 and 6 with Table 1). The mechanism of action of reserpine in inhibiting the amine uptake is also uncertain. In previous experiments, evidence has been presented that reserpine might interfere with a specific transport ATPase located in the adrenal granular membranes (Taugner & Hasselbach, 1966). If this were the case, one would have to postulate that two or more ATPases with different reserpine sensitivity are involved in the transport of the various amines.

In conclusion, membranes of bovine adrenal chromaffin granules take up amines other than those contained physiologically in these organelles by a specific transport mechanism. However, the magnitude of this uptake and the degree of its inhibition by reserpine differ for the various amines. The transport at the level of the granular membranes together with other mechanisms (e.g. intragranular aggregation, transport through the neuronal membrane) may be an important factor in determining the intraneuronal accumulation of physiological and false neurotransmitters.

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ROLE OF EXTRANEURONAL MECHANISMS IN THE TERMINATION OF CONTRACTILE RESPONSES TO AMINES IN VASCULAR TISSUE

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1 The role of the uptake and release of agonist from extraneuronal sites in the termination of responses of rabbit aortic strips to amines was studied.

2 Strips were contracted with adrenaline or noradrenaline and after a response plateau was reached, the muscle chambers were washed free of agonist and the relaxation in Krebs solution recorded. After inhibition of catechol-*O*-methyl-transferase, monoamine oxidase and neuronal uptake the relaxation rate was greatly prolonged. Evidence is provided that this very slow relaxation resulted from the accumulation of intact amine at extraneuronal sites during exposure to the agonist and its subsequent release past receptors due to a reversal of the concentration gradient after washout.

3 Pretreatment with the haloalkylamine, GD-131 (*N*-cyclohexylmethyl-*N*-ethyl-β-chloroethylamine), an inhibitor of extraneuronal uptake, returned the slow relaxation rate after enzyme inhibition towards that of control strips. By blocking the extraneuronal transport of amines their accumulation at intracellular loci after enzyme inhibition was prevented.

4 The effects of GD-131 and 17β-oestradiol on the relaxation rate of untreated strips contracted by adrenaline and noradrenaline confirmed that extraneuronal uptake to sites of enzymatic activity is the major mechanism terminating their action.

5 Inactivation of extraneuronal transport sites by GD-131 was prevented by protecting them with 17β-oestradiol or normetanephrine during exposure to the haloalkylamine, pointing to a common site of action of these agents on a specific carrier system for amines.

6 Evidence is presented that the relaxation from contractions induced by histamine and 5-hydroxytryptamine also involves extraneuronal accumulation and release, probably by an uptake process which is identical to the one for catecholamines.

Introduction

An analysis of sensitization of response magnitudes and of relaxation rates of aortic strips after oil immersion has led to the conclusion that the major process terminating the action of noradrenaline and adrenaline in vascular tissue is uptake and distribution in effector cells (Kalsner, 1966; Kalsner & Nickerson, 1969a). It was stated in the latter publication that, although distribution in cell water is the immediate event which terminates the action of these catecholamines, 'the capacity of this process appears to be limited, and its contribution to the body economy is dependent on the associated enzymic processes for definitive inactivation of the mediator'.

The rate of relaxation of aortic strips after washout of a stimulant drug from the muscle chambers has been shown to be a valid and sensitive index of the declining concentration of agonist at the pertinent tissue receptors (Kalsner & Nickerson, 1968a, b). The dissociation of diffusion of agonist out of the tissue from intrinsic inactivation is achieved by replacing the aqueous bathing medium with mineral oil after a steady-state contraction in response to the agonist under study has been established. Under these conditions, relaxation is a direct measure of termination of action by intrinsic mechanisms. In contrast, relaxation in Krebs solution permits movement of agonist out of the tissue into the external bathing medium to function along with intrinsic mechanisms of disposition in terminating action.

The present experiments are based on an analysis of relaxation rates of aortic strips in Krebs solution in order to assess the involvement of uptake and release from extraneuronal sites in terminating responses to moderate concentrations of catecholamines and to define more clearly the nature of these sites and their possible function in terminating contractions to other biogenic amines.

Methods

Helically cut strips of rabbit thoracic aorta about 2.5 x 23 mm were prepared for isotonic recording as described previously (Kalsner & Nickerson, 1968a). All experiments were done at 37°C and the strips were kept under a tension of 2 grams. The strips were allowed to equilibrate in the muscle chambers for at least 90 min before drug testing. The muscle chambers were of 15 ml working volume and contained a modified Krebs-Henseleit solution (mM. NaCl, 115.3; KCl, 4.6, $CaCl_2$, 2.3; $MgSO_4$, 1.1; $NaHCO_3$, 22.1; KH_2PO_4, 1.1; and glucose, 7.8) with disodium edetate added to give a final concentration of 0.03 mM.

All solutions were made with double-distilled water which had been passed through a cation-exchange column. Final concentrations of drugs in the muscle chambers are expressed in terms of molarity and as g/ml (w/v). Concentrations of (−)-noradrenaline and (−)-adrenaline bitartrates, methoxamine hydrochloride, metaraminol bitartrate, histamine dihydrochloride, 5-hydroxytryptamine creatinine sulphate, U-0521 (3,4,dihydroxy-2-methyl propiophenone) and iproniazid were calculated as the free base and cocaine hydrochloride, amodiaquin hydrochloride (Camoquin) and angiotensin amide as the salt.

GD-131 (*N*-cyclohexylmethyl-*N*-ethyl-β-chloroethylamine) was prepared on the day of use in a concentration of 1 mg/ml in propylene glycol to which a few drops of 0.01 N HCl was added. All solutions of sympathomimetic amines, 5-hydroxytryptamine and histamine contained 0.01 N HCl and were prepared fresh daily. Reserpine was dissolved in 10% ascorbic acid and rabbits were injected intramuscularly with 1 mg/kg about 18 h before death. This procedure has been shown to block completely the responses to tyramine ($7.3 \times 10^{-7} - 2.2 \times 10^{-5}$ M; 0.1 − 3.0 μg/ml) a potent releaser of endogenous catecholamines in aortic strips (Kalsner, 1970b).

Aortic strips were exposed to a stimulant drug for a sufficient period, usually 20 min, to allow the contractions to reach a stable plateau before washout. Washout of an agonist drug from the muscle chambers involved three initial changes of the bath fluid, followed by one change every 5 min until relaxation was complete. The chambers were washed by overflow, to minimize any mechanical artifact during the washout procedure. The percentage relaxation at any time was calculated from the ratio of the residual amplitude of contraction to that immediately before washing the stimulant drug from the chamber. To compare the rates of relaxation the time required for each strip to relax 50% was measured and the mean time calculated for each treatment. Details of this procedure were given previously (Kalsner & Nickerson, 1968a, b) Mean values are reported with their standard errors. Differences between means were compared by Student's *t* test and those with *P* values of 0 05 or less were considered significant.

Several treatment conditions were employed to investigate mechanisms for the inactivation of agonists. In a majority of experiments strips cut from the same aorta were utilized for all treatment groups with a given agonist to reduce variability.

(A) *Control*

The agonist was added to the muscle chambers at the required concentration and 20 min later they were washed as indicated above and the relaxations were recorded. In the case of histamine, exposure to the agonist was usually for a total of 10 min prior to washout.

(B) *Cocaine*

Strips were exposed to cocaine (2.9×10^{-5} M; 10 μg/ml) 10 min before contraction with the agonist to inhibit neuronal uptake (Kalsner & Nickerson, 1969b) and about 20 min later the chambers were washed with Krebs solution containing cocaine (2.9×10^{-5} M).

(C) *Iproniazid*

Strips were exposed to iproniazid (5.6×10^{-4} M; 100 μg/ml) for 30 min, the drug was washed out and an additional 30 min, with frequent washes, was allowed to elapse before the addition of the agonist. The strips were then treated in the same way as controls. This procedure was shown previously to produce essentially complete inhibition of monoamine oxidase and diamine oxidase but to produce no other alteration in the responsiveness of the tissue or in the intrinsic disposition of amines (Kalsner & Nickerson, 1968a, b, Kalsner, 1970a).

(D) *Iproniazid plus U-0521 or amodiaquin*

Strips were exposed to iproniazid as in C and then to either U-0521 (5.3×10^{-5} M; 10 μg/ml) or to amodiaquin (6.5×10^{-6} M; 3 μg/ml) for 10 min and without washout, contracted by the agonist as in A. The muscle chambers were washed with Krebs solution containing U-0521 (5.3×10^{-5} M) or amodiaquin (6.5×10^{-6} M). The specificity of action and completeness of the procedures used to inhibit catechol-*O*-methyltransferase and imidazole-*N*-methyltransferase with U-0521 and amodiaquin, respectively, have been described previously (Kalsner, 1969a, b, Kalsner, 1970a, b).

(E) *Iproniazid plus U-0521 plus cocaine*

Strips were pretreated with iproniazid as in C and then exposed to U-0521 (5.3×10^{-5} M) and to cocaine (2.9×10^{-5} M) followed 10 min later, without washout of the muscle chambers, by the agonist as in A. The chambers were washed with Krebs solution containing both U-0521 and cocaine.

(F) *GD-131*

Strips were exposed to GD-131 (1.3×10^{-5} M; 3 μg/ml) for 15 min followed by a minimum period of 15 min with frequent washes of the muscle chambers and then treated in the same way as the controls. This haloalkylamine compound has been shown to block persistently the uptake of catecholamines in vascular effector cells and to do so at a concentration well below that which blocks the α-receptors (Kalsner & Nickerson, 1969a).

(G) *17β-oestradiol*

Strips were exposed to 17β-oestradiol (3.7 or 11×10^{-5} M; 10 or 30 μg/ml) for 15 min and without washout of the muscle chambers contracted by the agonist for 20 min and treated as in A. This steroid and several others have been reported to block the extraneuronal inactivation of catecholamines in vascular tissue (Kalsner, 1969a, b), a finding which has been confirmed by others for cardiac tissue and established as due to blockade of uptake (Salt, 1972).

(H) *Iproniazid plus GD-131 (or 17β-oestradiol) plus U-0521 plus cocaine*

Strips were pretreated with iproniazid as in C and exposed to GD-131 as in F (or 17β-oestradiol as in G) and then treated with U-0521 plus cocaine as in E. The chambers were washed with Krebs solution containing U-0521 plus cocaine.

(I) *Iproniazid plus GD-131*

Strips were pretreated with iproniazid as in C and with GD-131 as in F and then treated in the same way as group A.

(J) *Iproniazid plus amodiaquin plus GD-131*

Strips were pretreated with iproniazid as in C and with GD-131 as in F and then exposed to amodiaquin (6.5×10^{-6} M) followed 10 min later, without washout of the muscle chambers, by the agonist as in A The chambers were washed with Krebs solution containing amodiaquin (6.5×10^{-6} M).

(K) *Protection series*

After treatment with iproniazid as in C, strips were exposed to normetanephrine (5.5 or 16×10^{-5} M; 10 or 30 μg/ml) or 17β-oestradiol (3.7×10^{-5} M) for 10 min and without washout to GD-131 (1.3×10^{-5} M) for an additional 15 minutes. The drugs were washed out of the muscle chambers and 120 min later, after exposure to U-0521 and to cocaine as in E, they were contracted by adrenaline as in A. Control strips were treated identically except for the omission of normetanephrine or 17β-oestradiol and/or GD-131.

Results

Relaxation of adrenaline- and noradrenaline-contracted strips in Krebs solution

Aortic strips contracted by a low concentration of adrenaline (1.6×10^{-7} M; 30 ng/ml) reached a mean response amplitude of 36.7 ± 1.4 mm, about half maximal, and relaxed 50% in a mean of 1.4 min after washout of the agonist from the muscle chambers. This process of relaxation towards the pre-contraction baseline reflects the rapidly declining concentration of agonist in the vicinity of receptors due to efficient intrinsic processes of inactivation and also removal by diffusion into the surrounding bathing medium (Kalsner & Nickerson, 1968a, b) (Figure 1a, Table 1). As shown in Table 1, inhibition of neuronal uptake alone, with cocaine, only had a slight effect on the relaxation rate of strips contracted by adrenaline whereas inhibition of extraneuronal uptake with GD-131 produced a material slowing of relaxation, reflecting the importance of this latter process in termination of action, even in the presence of a competing gradient of diffusion of the agonist into the external bathing medium (Table 1, Figure 1).

After direct inhibition of catechol-*O*-methyltransferase and monoamine oxidase with U-0521 and iproniazid the relaxation time of adrenaline-contracted strips was prolonged strikingly to a mean of 20.3 min (Table 1, Figure 1). Additional pretreatment of strips with cocaine had no significant effect on this slow relaxation. The finding that block of neuronal uptake did not speed significantly the relaxation of strips in which

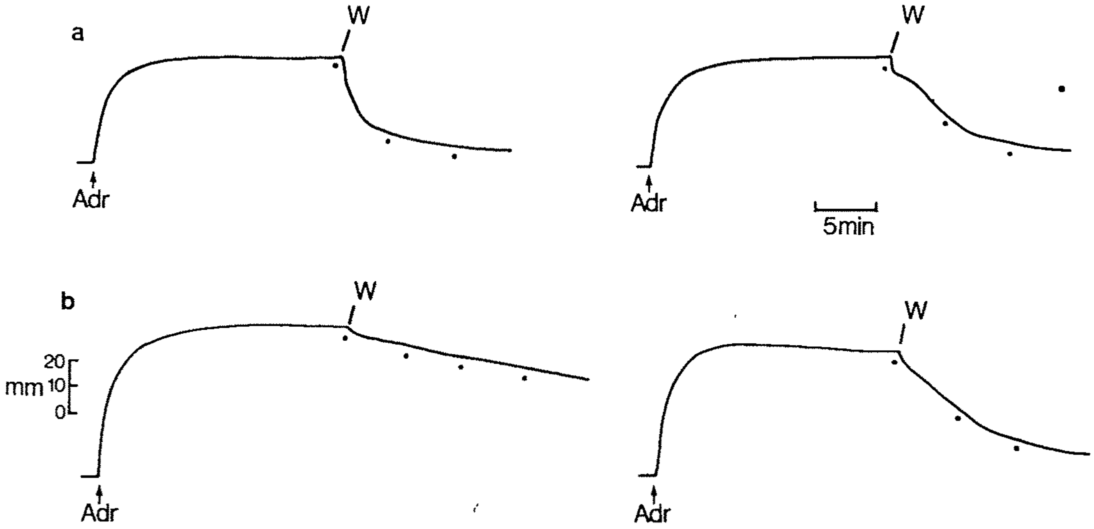

Figure 1 Relaxation in Krebs solution of aortic strips contracted by adrenaline (Adr) (1.6×10^{-7} M). (a) Left, control contraction in response to adrenaline and relaxation in Krebs; right, strip from the same aorta pretreated with GD-131 before contracted by adrenaline; (b) left, strip pretreated with iproniazid and exposed to U-0521 and cocaine before contracted by adrenaline; right, strip from the same aorta pretreated with iproniazid and GD-131 and exposed to U-0521 and cocaine before contracted by adrenaline. Dots indicate washout of the muscle chambers every five minutes. Initial washout is also indicated by (W).

Table 1 Relaxation in Krebs solution of aortic strips contracted by adrenaline and noradrenaline

Agonist	*Treatment*	*No. of strips*	*Time to relax 50%*
Adrenaline 1.6×10^{-7} M	Untreated	18	1.4 ± 0.1
		6	1.9 ± 0.2‡
	Cocaine	12	2.3 ± 0.3*
	Iproniazid plus U-0521	4	20.3 ± 0.6*
	Iproniazid plus U-0521 plus cocaine	23	18.8 ± 1.0*
		7	21.7 ± 1.3*‡
	GD-131	11	4.0 ± 0.3*
		4	6.0 ± 0.7*‡
	GD-131 plus cocaine	4	6.0 ± 0.6†
	17β-Oestradiol	8	4.7 ± 0.9*
	Iproniazid plus GD-131 plus U-0521 plus cocaine	16	4.6 ± 0.3†
		3	7.2 ± 0.3†‡
	Iproniazid plus 17β-oestradiol plus U-0521 plus cocaine	12	5.9 ± 0.7†
Noradrenaline (1.8×10^{-7} M)	Untreated	8	2.4 ± 0.4
	Cocaine	6	3.4 ± 0.2
	Iproniazid plus U-0521	4	15.8 ± 2.7*
	Iproniazid plus U-0521 plus cocaine	10	17.8 ± 2.7*
	GD-131	8	4.0 ± 0.3*
	Iproniazid plus GD-131 plus U-0521 plus cocaine	6	4.8 ± 0.9†

* Significantly different from untreated group.

† Significantly different from corresponding group without GD-131 or 17β-oestradiol treatment.

‡ Values from reserpine-pretreated preparations. In these cases, the indicated statistical comparisons are with other reserpine pretreated groups.

enzymic inactivation was inhibited (Table 1) served to exclude the possibility that uptake and subsequent release from neuronal structures acts as a mechanism prolonging relaxation after enzyme inhibition. Therefore, the likelihood that the slow relaxation of aortic strips after direct inhibition of *O*-methylation and deamination is the consequence of an accumulation and subsequent release of adrenaline from extraneuronal sites onto receptors was investigated with the aid of GD-131.

After inhibition of intrinsic pathways of inactivation, strips exposed to GD-131 and subsequently contracted by adrenaline relaxed much faster than their counterparts without GD-131 pretreatment (Figure 1b, Table 1). In fact, the mean relaxation times of strips pretreated with the haloalkylamine in the presence or absence of functional intrinsic pathways of disposition did not differ significantly, as would be anticipated if extraneuronal uptake to intracellular loci were blocked in both conditions If penetration to sites of enzymatic inactivation is blocked then the functional status of the enzymes beyond the barrier is irrelevant.

GD-131 in the concentration and time of exposure used here does not block materially uptake into neuronal structures (Kalsner & Nickerson, 1969a) and this is demonstrated in the present experiments by the finding that the combination of cocaine and GD-131 had an approximately additive effect on the relaxation rate (Table 1). It was also noted in experiments with GD-131 that the relaxation curve of strips contracted by adrenaline 30 min or 3 h after a 15 min exposure to the antagonist did not differ significantly, pointing to the formation of a stable chemical bond between the haloalkylamine and some tissue component, as had been suggested previously (Kalsner & Nickerson, 1969a).

Responses to adrenaline were significantly increased by reserpine treatment, reaching a mean of 59.0 ± 6 0 mm in six control strips, and this may account for the slight but consistent decrease in the absolute relaxation rates in these groups as shown in Table 1 (Kalsner & Nickerson, 1969c). However, the results obtained, and the relative effects of the inhibitors, did not differ materially from those recorded in untreated preparations, making it highly improbable that endogenous catecholamine stores are, in any way, involved in the observed relaxation rates (Table 1). In other experiments, strips were contracted with noradrenaline (1.8×10^{-7} M; 30 ng/ml) and the relaxations recorded. The mean amplitude of contraction of control strips was 31.9 ± 3.0 mm. Although there were some differences in the absolute relaxation rates, the effects of inhibitors of intrinsic mechanisms of disposition and of GD-131 were similar to those observed in experiments with adrenaline (Table 1). The specificity of the effects of GD-131 on relaxation rates is indicated by the finding that the relaxation from contractions in response to angiotensin (9.7×10^{-9}M; 10 ng/ml) was not modified by treatment with the haloalkylamine (Table 2, Figure 2b)

Further evidence that the effects of GD-131 are related to blockade of extraneuronal uptake was obtained in experiments with the inhibitor 17β-oestradiol. The relaxation rate of untreated strips contracted by adrenaline (1.6×10^{-7} M) was significantly slowed by treatment of strips with 17β-oestradiol but after inhibition of intrinsic mechanisms of disposition the relaxation time was much reduced by the steroid (Table 1) These are effects similar to those of GD-131, and again are explicable on the basis of inhibition of extraneuronal uptake sites.

Other sympathomimetic amines

Methoxamine is not a substrate for monoamine oxidase or catechol-*O*-methyltransferase and is not taken up into nerve endings in vascular tissue (Kalsner & Nickerson, 1968a). For this reason any extraneuronal uptake of this amine should lead to its accumulation intact and subsequent release past receptors after washout. Blockade of uptake would therefore speed relaxation. The effect of inhibition of extraneuronal uptake on the rate of relaxation from contractions elicited by this agonist was explored. For these experiments a concentration of at least 2.4×10^{-6} M (0.5 μg/ml) of methoxamine was required in order to obtain a sufficient amplitude of response for the study of relaxation rates. The mean contraction amplitudes to $2 4 \times 10^{-6}$ M and 4.7×10^{-6} M were 34.3 ± 0.9 and 40.5 ± 2.4 mm. As shown in Table 2 the relaxation rates of strips contracted by methoxamine with and without pretreatment with GD-131 did not differ significantly. These experiments were duplicated over a range of methoxamine concentrations in reserpine-pretreated preparations where the results showed a slight but significant effect of GD-131 in hastening relaxation (Table 2).

Metaraminol is not metabolized by catechol-*O*-methyltransferase or monoamine oxidase but it is accumulated by sympathetic nerve terminals in cardiac tissue (Iversen, 1965). Aortic strips were contracted with metaraminol (6×10^{-6} M; 1 μg/ml) with or without prior exposure to GD-131 or to cocaine (2.9×10^{-5} M) as described in the methods section, and 20 min later the muscle chambers were washed and relaxation rates recorded. The mean contraction amplitude of

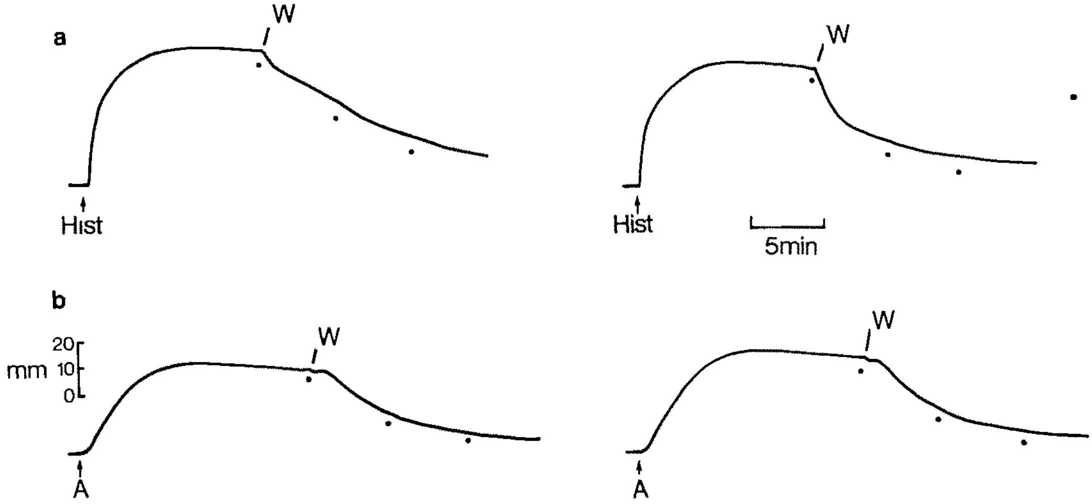

Figure 2 Relaxation in Krebs solution of aortic strips contracted by histamine (Hist) (5.4×10^{-5} M) and angiotensin (A) (9.7×10^{-9}M). (a) Left, control contraction in response to histamine and relaxation in Krebs; right, strip from the same aorta pretreated with GD-131 before contracted by histamine, (b) left, control contraction in response to angiotensin and relaxation in Krebs; right, strip from the same aorta pretreated with GD-131 before contracted by angiotensin. Initial washout of the chambers is indicated by (W) and dots indicate washes every five minutes.

control strips was 37.4 ± 1.8 mm. Cocaine alone had no clear effect on the rate of relaxation, but the rate was increased significantly by GD-131. The combination of the two agents had no greater effect than did GD-131 alone. It appears that the accumulation of metaraminol in sympathetic nerve endings in vascular tissue is not a significant factor affecting relaxation, but that some slight extraneuronal accumulation and release is involved.

Other biogenic amines

The possibility that agonists acting on receptors other than those for sympathomimetic amines also

Table 2 Relaxation in Krebs solution of aortic strips contracted by sympathomimetic amines and angiotensin

Agonist	*Treatment*	*No. of strips*	*Time to relax 50%*
Methoxamine (2.4×10^{-6} M)	Untreated	4	5.4 ± 0.5
	GD-131	4	5.1 ± 0.3
Methoxamine (4.7×10^{-6} M)	Untreated	12	8.9 ± 0.8
		8	8.8 ± 0.7†
	GD-131	12	8.2 ± 0.5
		8	6.7 ± 0.5*†
Methoxamine (4.7×10^{-5} M)	Untreated	5	18.0 ± 0.6†
	GD-131	5	13.6 ± 0.8*†
Metaraminol (6×10^{-6} M)	Untreated	15	5.3 ± 0.6
	Cocaine	18	4.9 ± 0.4
	GD-131	16	3.7 ± 0.3*
	GD-131 plus cocaine	13	4.3 ± 0.2*
Angiotensin (9.7×10^{-9} M)	Untreated	4	5.2 ± 0.5
	GD-131	4	5.2 ± 0.6

* Significantly different from untreated group.
† Values from reserpine-pretreated preparations.

make use of the extraneuronal uptake process to reach intracellular loci and that this affects relaxation was examined. Strips were contracted with histamine (2.7 or 5.4 x 10^{-5} M; 3 or 6 μg/ml) with and without prior exposure to GD-131 and the relaxation rates recorded after washout of the chambers. The mean contraction amplitudes at the two concentrations did not differ significantly; they were 46.0 ± 5.0 and 42.0 ± 2.4 mm, respectively. GD-131 increased the relaxation rate at both concentrations of histamine, although the effect at the lower concentration (2.7 x 10^{-5} M) was not statistically significant, probably because of the small number of values (Table 3, Figure 2a). Other strips were contracted with histamine, with and without haloalklylamine pretreatment, after inhibition of imidazole-*N*-methyltransferase and diamine oxidase. It was reported previously that these enzymes are chiefly responsible for inactivating histamine in vascular tissue (Kalsner, 1970a). Inhibition of *N*-methylation and deamination with amodiaquin and iproniazid slowed slightly, but not significantly, the rate of relaxation but after the additional inhibition of extraneuronal uptake with GD-131, the rate of relaxation was significantly increased and comparable with that of control strips treated with the haloalkylamine (Table 3). 17β-Oestradiol had an effect on relaxation after enzyme inhibition similar to that of GD-131. The rate of relaxation after contractions induced by 5-hydroxytryptamine (5.7 x 10^{-6} M; 1 μg/ml), which reached a mean amplitude of 30.3 ± 2.5 mm, was also significantly increased by GD-131 in control and reserpine-treated strips after inhibition of intracellular inactivation by monoamine oxidase (Table 3).

Protection of extraneuronal uptake sites against inactivation by GD-131

Attempts were made to protect the extraneuronal transport sites for sympathomimetic amines against inactivation by GD-131. Aortic strips were exposed to GD-131 in the presence and absence of normetanephrine (5.5 or 16 x 10^{-5} M; 10 or 30 μg/ml), a compound reported to have high affinity for the extraneuronal uptake sites of cardiac tissue (Iversen, 1965). After inhibition of enzymatic inactivation and neuronal uptake the strips were contracted with adrenaline (1.6 x 10^{-7} M) and the relaxation rates recorded Strips exposed to GD-131 in the presence of the higher concentration of normetanephrine (1.6 x 10^{-4} M) relaxed at a rate comparable with that of strips with no GD-131 treatment, indicating virtually complete protection of the transport sites by the amine (Table 4, Figure 3). The lower concentration of normetanephrine

Table 3 Relaxation in Krebs solution of aortic strips contracted by histamine and 5-hydroxytryptamine (5-HT)

Agonist	*Treatment*	*No. of strips*	*Time to relax 50%*
Histamine (2.7 x 10^{-5} M)	Untreated	3	5.0 ± 0.4
	Iproniazid plus amodiaquin	12	7.7 ± 1.1
	GD-131	2	1.5 ± 0.3
	Iproniazid plus GD-131 plus amodiaquin	7	3.5 ± 0.4†
	Iproniazid plus 17β-oestradiol plus amodiaquin	3	1.7 ± 0.1†
Histamine (5.4 x 10^{-5} M)	Untreated	5	5.8 ± 0.4
	Iproniazid plus amodiaquin	5	6.6 ± 0.5
	GD-131	4	2.8 ± 0.4*
	Iproniazid plus GD-131 plus amodiaquin	5	3.1 ± 0.3†
5-HT (5.7 x 10^{-6} M)	Untreated	10	8.9 ± 0.5
		9	9.2 ± 0.7‡
	Iproniazid	11	15.7 ± 1.1*
		16	17.4 ± 1.6*‡
	GD-131	10	9.4 ± 0.3
		8	8.7 ± 0.4‡
	Iproniazid plus GD-131	12	11.8 ± 0.8†
		16	10.8 ± 0.5†‡

* Significantly different from untreated group.
† Significantly different from corresponding group without GD-131 or 17β-oestradiol treatment.
‡ Values from reserpine-treated preparations.

produced an intermediate level of protection which did not quite reach statistical significance ($P < 0.1 > 0.05$). In matching control strips, exposure to normetanephrine (5.5 or 16 x 10^{-5} M) alone had no significant effect on the relaxation curve of strips subsequently contracted by adrenaline, after inhibition of intrinsic mechanisms of disposition (Table 4). In other experiments, strips were exposed to GD-131 in the presence of 17β-oestradiol (3.7 x 10^{-5} M) as described in the methods section, and after inhibition of intrinsic pathways of inactivation, they were contracted by adrenaline and the relaxation curves recorded. The effectiveness of 17β-oestradiol in protecting against inactivation of extraneuronal uptake sites by GD-131 is demonstrated in Table 4 and Figure 3. In control strips treated identically with 17β-oestradiol, except for exposure to GD-131, the relaxation rate was increased slightly, but not significantly, compared to strips without 17β-oestradiol treatment, undoubtedly due to a

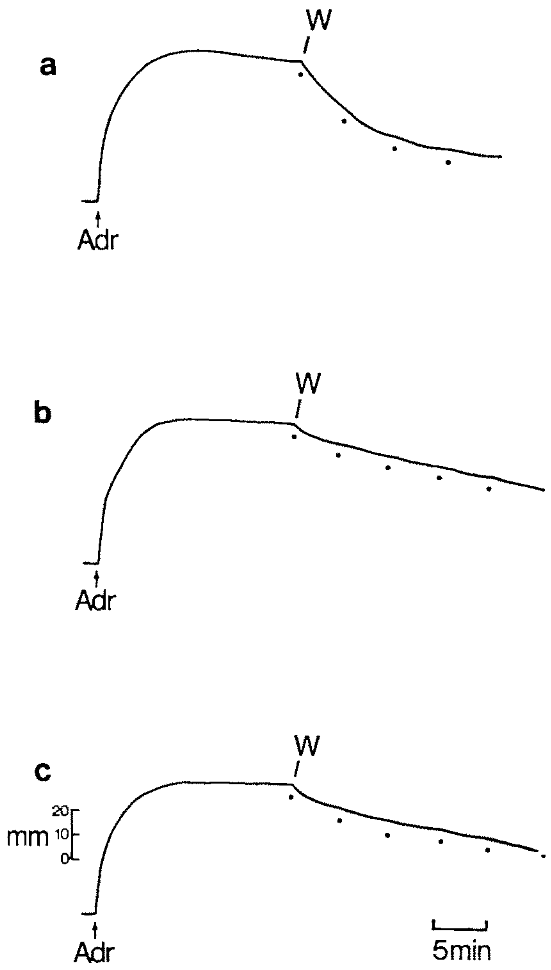

Figure 3 Protection against effects of GD-131 on relaxation of adrenaline (Adr) (1.6 x 10^{-7} M)-contracted strips. (a) Relaxation in Krebs solution of strip pretreated with iproniazid and GD-131 and subsequently exposed to U-0521 and cocaine and contracted by adrenaline; (b) strip treated as in (a) except that exposure to GD-131 was in the presence of normetanephrine (1.6 x 10^{-4} M); (c) strip treated as in (a) except exposure to GD-131 was in the presence of 17β-oestradiol (3.7 x 10^{-5} M) All strips were taken from the same aorta. Details of treatment given in text. Initial washout of the chambers is indicated by (W) and dots indicate washes every five minutes.

Table 4 Protection against effects of GD-131 on relaxation of adrenaline-contracted strips

Treatment	*Additional treatment*	*No. of strips*	*Time to relax 50%*
Iproniazid plus U-0521 plus cocaine	—	7	21.3 ± 2.0
Iproniazid plus U-0521 plus cocaine	GD-131	6	6.6 ± 1.1*
Iproniazid plus U-0521 plus cocaine	GD-131 in presence of NM (5.5 x 10^{-5} M)	3	10.7 ± 1.4*
Iproniazid plus U-0521 plus cocaine	GD-131 in presence of NM (1.6 x 10^{-4} M)	5	25.2 ± 1.0†
Iproniazid plus U-0521 plus cocaine	NM (5.5 x 10^{-5} or 1.6 x 10^{-4} M)	4	21.7 ± 0.4†
Iproniazid plus U-0521 plus cocaine	GD-131 in presence of 17β-oestradiol	6	18.9 ± 2.5†
Iproniazid plus U-0521 plus cocaine	17β-Oestradiol	3	16.8 ± 1.2†

Strips were exposed to GD-131 in the presence or absence of 17β-oestradiol or normetanephrine (NM). The drugs were washed out of the chambers and 120 min later, after inhibition of intrinsic pathways of disposition, the strips were contracted by adrenaline (1.6 x 10^{-7} M). Details of exposure to inhibitors and contraction by adrenaline are given in text.

* Significantly different from control group without additional treatment.

† Significantly different from group which received GD-131 alone as additional treatment.

residual effect of the steroid as an inhibitor of extraneuronal uptake, even 120 min after washout of the muscle chambers. The kymograph traces of a typical protection experiment are shown in Figure 3.

Discussion

The usually rapid relaxation in Krebs of aortic strips contracted by low to moderate concentrations of noradrenaline and adrenaline is prolonged strikingly after inhibition of enzymatic pathways of inactivation. It appears that amine which normally is transported into non-nervous structures and metabolized, is retained intact and accumulated at intracellular loci after enzyme inhibition, and when the agonist is cleared from the bathing medium by washout of the muscle chambers an outwardly directed concentration gradient is established. This movement of intact agonist past the receptors is responsible for the very slow decline of the response.

This interpretation was supported by the finding that the haloalkylamine, GD-131, returned the relaxation rates of treated strips towards control values. By blocking the inward transport of amines and their accumulation at intracellular loci after enzyme inhibition their consequent release past receptors, after washout of the muscle chambers, was prevented. The outward transport of amines also may be blocked by GD-131 but it is probably not relevant to the present discussion since, in all cases, treatment with the inhibitor preceded exposure to the amines.

The uptake and release of amine from neuronal structures could not account for the prolongation of the response to adrenaline and noradrenaline and the effects of GD-131. These experiments were performed in the presence of a concentration of cocaine (2.9×10^{-5} M) that blocks the neuronal membrane transport system virtually completely (Iversen, 1965). In addition, blockade of the nerve membrane transport system alone had only a slight effect on relaxation rates, whereas inhibition of monoamine oxidase and catechol-*O*-methyltransferase produced an effect comparable with that of combined inhibition of all three intrinsic processes of inactivation.

The prolongation by GD-131 of the relaxation time of untreated aortic strips contracted by adrenaline (1.6×10^{-7} M) or noradrenaline (1.8×10^{-7} M) is also explicable on the basis of a single known action of this haloalkylamine on amine transport across extraneuronal membranes. When enzyme systems are functional, extraneuronal uptake continues to act as a sink for agonist during the relaxation process as well as during exposure to the amine. By blocking the intracellular penetration of amines their volume of distribution in cell water is decreased and their metabolism prevented. Consequently, the effect of GD-131 on the relaxation rate of untreated strips represents accurately the role of extraneuronal uptake in terminating action; that is in removing amine from the region of the receptors after clearance of the muscle chamber by washout. The increase in relaxation times indicates that approximately 40% and 65% of noradrenaline and adrenaline are inactivated by extraneuronal uptake even in the presence of the competing pathway of diffusion into the muscle chambers. Uptake into neuronal structures had a lesser role in the inactivation of these amines based on an analysis of relaxation rates after cocaine, accounting maximally for 30% and 39%. The slight overlap of the effects of the two inhibitors in the case of adrenaline is probably related to the previously reported activity of cocaine as a weak inhibitor of extraneuronal uptake in aortic strips (Kalsner & Nickerson, 1968b; 1969a, b). The above interpretation is supported by the results of experiments with 17β-oestradiol, another known inhibitor of extraneuronal uptake, which had effects on relaxation rates similar to those of the haloalkylamine. In addition, it is consistent with data obtained previously using the technique of oil immersion (Kalsner & Nickerson, 1969a, b).

It thus appears reasonable to maintain that a slowing of relaxation after prior inhibition of extraneuronal uptake represents block of a site of loss and an increase in relaxation a block of the sequence of events of extraneuronal uptake, accumulation and release. Although there may be alternative explanations of certain of the present data, the analysis provided here is consistent with the known action of the haloalkylamines and steroids in blocking extraneuronal transport. No new actions need be invoked. In addition, since these experiments involve fluxes of agonists in the biophase during termination of action which is a physiological event, it is appropriate to use the responding tissue itself as probably the most sensitive index available of the declining agonist concentration in the region of the pertinent tissue receptors.

After completion of the present work, Trendelenburg (1974) published a study on the relaxation of aortic strips after contractions by adrenaline and noradrenaline. His work, using similar methods, demonstrated the importance of extraneuronal efflux in limiting the rate of relaxation after enzyme inhibition. However, Trendelenburg did not take into account the role of tissue sites as sites of loss during the relaxation process and although it is a complicating factor it is critical to a complete evaluation of the data.

Experiments of other workers using different techniques have also provided definitive evidence of an extraneuronal uptake process for noradrenaline in vascular tissue (Avakian & Gillespie, 1968; Gillespie, Hamilton & Hosie, 1970; Gillespie & Muir, 1970; Burnstock, McCulloch, Story & Wright, 1972). For example, Avakian & Gillespie (1968), using a histochemical technique, demonstrated fluorescence of the smooth muscle cells of the rabbit ear artery after exposure to a high concentration of noradrenaline (100 μg/ml) which was prevented by pretreatment of the tissue with the haloalkylamine antagonist phenoxybenzamine. In contrast, surface binding of noradrenaline to collagen or elastic tissue was not reduced by such treatment. In another approach, Nedergaard & Bevan (1971) demonstrated a phenoxybenzamine-sensitive uptake of [^{3}H]-noradrenaline (10^{-5} M) into the media of rabbit aorta.

The experiments described here show the influence on relaxation rates of extraneuronal uptake of both noradrenaline and adrenaline at the probably physiological concentrations of 1.8 and 1.6×10^{-7} M, concentrations which produce about half-maximal contractions of aortic strips. It is clear from the present experiments that extraneuronal uptake has a key role in transporting even these low concentrations of adrenaline and noradrenaline to sites of inactivation in effector cells and that the usual fate of the transported amine is inactivation by *O*-methylation and deamination.

The work of other investigators suggests that a carrier system is involved in the extraneuronal transport of noradrenaline in vascular tissue (Gillespie *et al.*, 1970; Gillespie & Towart, 1973) and the present experiments provide strong support for a specific carrier site for biogenic amines which is inhibited competitively by GD-131 and 17β-oestradiol. Blockade of the extraneuronal transport of amines by GD-131 could be prevented by incubation of the tissue with the haloalkylamine in the presence of normetanephrine. Thus, in all probability, GD-131 combines with the same site that is used to transport the amines. Similarly, 17β-oestradiol protects effectively against inactivation of the transport mechanism by the haloalkylamine, pointing again to a common site of action of all these agents.

The present data indicate that the extraneuronal uptake process is not specific for adrenaline and noradrenaline in the control of relaxation rates but serves also to transport other biogenic amines to intracellular loci of enzymatic inactivation and storage. GD-131 speeded the relaxation of strips contracted by histamine and 5-hydroxytryptamine, after inhibition of intrinsic pathways of disposition, results similar to those obtained with the catecholamines. The relaxation of untreated strips contracted by histamine was also increased by the haloalkylamine indicating that extraneuronal accumulation of intact histamine occurs even in the presence of functional pathways of inactivation, and at a concentration of histamine which produces responses of only moderate amplitude in aortic strips. 17β-Oestradiol also increased the relaxation rate of strips contracted by histamine confirming the previous report that this steroid inhibits the extraneuronal inactivation of histamine in vascular tissue (Kalsner, 1970a). The finding that extraneuronal uptake plays a critical role in the inactivation of a variety of amines in vascular tissue suggests that derangement of the extraneuronal transport system should be considered as a possible factor in experimental and clinical conditions in which enhanced responses to or altered metabolism of biogenic amines are observed.

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STUDIES ON THE MECHANISM OF ACTION OF A NEW Ca^{2+} ANTAGONIST, 8-(*N,N*-DIETHYLAMINO)OCTYL 3,4,5-TRIMETHOXYBENZOATE HYDROCHLORIDE IN SMOOTH AND SKELETAL MUSCLES

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1 The rabbit aortic strip, guinea-pig ileum and rabbit skeletal muscle sarcoplasmic reticulum preparations were used to determine at which sites and in what manner 8-(*N,N*-diethylamino)-octyl 3,4,5-trimethoxybenzoate (TMB-8) interferes with Ca^{2+} availability in smooth and skeletal muscles.

2 TMB-8 (50 μM) significantly inhibited equivalent responses of the rabbit aortic strip to KCl and noradrenaline.

3 TMB-8 (65 μM) produced no significant alteration in the extracellular space of the guinea-pig ileum as measured with [^{3}H]-sorbitol.

4 The resting cellular Ca^{2+} influx as well as the resting $^{45}Ca^{2+}$ efflux in the guinea-pig ileum preparation were significantly inhibited by TMB-8 (65 μM).

5 TMB-8 (5 μM and 50 μM) had no significant effect on the uptake of $^{45}Ca^{2+}$ by the sarcoplasmic reticulum preparation of skeletal muscle; however, TMB-8 (5 μM) did significantly inhibit the caffeine (20 mM)-induced release of $^{45}Ca^{2+}$ from this preparation.

6 It is concluded that TMB-8 reduces Ca^{2+} availability in smooth and skeletal muscles by stabilizing Ca^{2+} binding to cellular Ca^{2+} stores and thereby inhibits the release of this Ca^{2+} by contractile stimuli.

Introduction

Recent studies of 8-(*N,N*-diethylamino)octyl 3,4,5-trimethoxybenzoate (TMB-8) have shown this agent to be a potent inhibitor of the contractility of both smooth (Malagodi & Chiou, 1974a) and skeletal (Malagodi & Chiou, 1974b) muscles. While TMB-8 has been shown to inhibit responses to all contractile agents tested in smooth and skeletal muscle (acetylcholine, 1,1-dimethyl-4-phenylpiperazinium, nicotine, noradrenaline, K^+, Ba^{2+} and caffeine) as well as responses to indirect and direct electrical excitation, the only instances of competitive antagonism of contractile responses were in studies of Ba^{2+}-induced contractions in smooth muscle and caffeine-induced contractions in skeletal muscle. Because both Ba^{2+} and caffeine are Ca^{2+}-releasing agents (Caldwell & Walster, 1963; Karaki, Ikeda & Urakawa, 1967; Weber & Herz, 1968; Thorpe & Seeman, 1971; Saito, Sakai & Urakawa, 1972; Antonio, Rocha e Silva & Yashuda, 1973; Thorpe, 1973), the competitive antagonism of responses to these agents suggests a specific interference of Ca^{2+}-release by TMB-8. Further, the antagonism of TMB-8 inhibition of responses of smooth and skeletal muscles to K^+ by increased extracellular Ca^{2+} concentrations provides additional evidence that TMB-8 exerts its inhibitory action by interfering with the availability of Ca^{2+} in the contractile sequence.

In smooth muscle, the Ca^{2+} involved in the contractile process appears to come both from intracellular and extracellular sources (Nayler, 1966; Ebashi, Endo & Ohtsuki, 1969; Somlyo, 1972; Holman, 1973), while Ca^{2+} supplied to the contractile proteins of skeletal muscle appears to come chiefly from the intracellularly-located sarcoplasmic reticulum (Nayler, 1966; Ebashi & Endo, 1968). The present study has been conducted in order to determine at which of these sites and in what manner TMB-8 interferes with the availability of Ca^{2+} in smooth and skeletal muscles.

Methods

Materials

8-(*N,N*-diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride (TMB-8) was synthesized as described previously (Malagodi & Chiou, 1974a). Other chemical agents used in this study include noradrenaline bitartrate (Winthrop Labs), caffeine sodium benzoate (Parke, Davis & Co.), $[^{45}Ca]Cl_2$ (specific activity, 955 mCi/mmol) (New England Nuclear), D-sorbitol-[1-^{3}H] ([^{3}H]-sorbitol, specific activity, 6.8 Ci/mmol) (New England Nuclear), NCS Solubilizer (Amersham/Searle Corp.), Aquasol scintillation solution (New England Nuclear), naphthalene (Eastman Organic Chemicals), 1,4-dioxane (W.H. Curtin & Co.), 2,5-diphenyloxazole (PPO) (New England Nuclear) and adenosine 5′-triphosphate, disodium salt (ATP) (Sigma Chemical Co.).

Rabbit aortic strip preparation

New Zealand white rabbits, 1.5-2.0 kg, were stunned by a blow on the head and decapitated. The thoracic aorta was removed and cut into circumferential strips 2-3 mm in width according to the method of Haeusler (1972). Each strip was mounted with 1 g of initial tension on a superfusion apparatus. Tyrode solution of the following composition (mM): NaCl, 137; KCl, 2.7; $MgSO_4$, 1.0; NaH_2PO_4, 0.36; $NaHCO_3$, 11 9; $CaCl_2$, 1.35 and dextrose, 11.1 was used. It was maintained at 37° C, oxygenated with 95% O_2 and 5% CO_2 and was pumped over the preparation by a Polystaltic pump (Buchler Instruments) at a constant flow rate of 6-7 ml/minute. The tension developed by the muscle during contraction was recorded on a Physiograph (Four-A, Narco Bio-Systems, Inc.). TMB-8 (50 μM) inhibition of muscle response to KCl (90 mg) and noradrenaline (0.1 μg), which were injected into the stream of superfusate solution, was examined. These particular doses of KCl and noradrenaline were selected because they produced equivalent responses which were approximately 50% of the maximum responses of the tissue to each agent. Responses to KCl and noradrenaline were examined on the same muscle before and during superfusion with TMB-8. The muscles were superfused with TMB-8 for 20 min before the repetition of KCl and noradrenaline responses. Only the phasic portion of noradrenaline responses was measured in this study.

Guinea-pig ileum preparation

Measurement of extracellular space. Guinea-pigs of either sex weighing 200-400 g, were used. The ileum was removed, cleaned with warm Tyrode solution, opened along its longitudinal axis and cut into 40 pieces, each approximately 0.5 cm in length. Twenty pieces each were used to measure the uptake of [^{3}H]-sorbitol in the absence and in the presence of TMB-8 and were placed in two 50 ml beakers containing 25 ml of Tyrode solution. Both Tyrode solutions were maintained at 37° C and oxygenated with 95% O_2 and 5% CO_2 throughout the experiment. After an initial 10 min equilibration period, TMB-8 was added to the experimental beaker to give a final concentration of 65 μM. This concentration of TMB-8 was just sufficient to produce 100% inhibition of responses to all contractile agents in this preparation. The incubation of both the experimental and control beakers was continued for an additional 20 minutes. At the end of this 20 min period, the solutions in both beakers were carefully and simultaneously decanted. Tyrode solution (25 ml) containing [^{3}H]-sorbitol (0.12 μCi/ml, final concentration) was then added to the control beaker and Tyrode solution (25 ml) containing the same amount of [^{3}H]-sorbitol plus TMB-8 (65 μM) was added to the experimental beaker. The time of addition of the [^{3}H]-sorbitol-Tyrode solutions was designated as zero time. After 1 min, two pieces each of ileum from the control solution and from the experimental solution were removed, blotted once on filter paper and weighed. The samples were then placed in scintillation vials containing 0.5 ml of NCS Solubilizer and agitated in a water bath at 50° C overnight in order to solubilize the tissues. This procedure was also carried out after incubation times of 5, 10, 20, 30, 45 and 120 minutes. After solubilization, the vials were removed from the water bath, cooled and 0.015 ml of glacial acetic acid was added to each vial to neutralize the solubilized tissue. Ten ml of scintillation solution (naphthalene, 100 g, and PPO, 7.0 g/litre 1,4-dioxane) was then added and the samples were placed in the dark for one week to eliminate chemiluminescence of the samples. The samples were then counted in a Beckman Scintillation System (Model 1650). Also counted with the tissue samples were four vials containing 10 ml of the scintillation solution plus 0.1 ml of the control [^{3}H]-sorbitol-Tyrode solution sampled at zero time (two vials) or 0.1 ml of the experimental [^{3}H]-sorbitol-Tyrode solution plus TMB-8 sampled also at zero time (two vials). To correct for quenching produced by the solubilized tissue, two additional 0.1 ml samples, each from the control and experimental [^{3}H]-sorbitol-Tyrode solutions, sampled at zero time, were added to scintillation solution containing tissue that had been solubilized previously. The results were expressed in terms of [^{3}H]-sorbitol spaces and were calculated according to the following equation:

$$\frac{\text{ct min}^{-1}\ \text{kg}^{-1}\ \text{(wet wt) of tissue}}{\text{ct min}^{-1}\ \text{ml}^{-1}\ \text{of solution}} = \frac{\text{ml of solution}}{\text{kg (wet wt) of tissue}}$$

Measurement of Ca^{2+} movements The experimental protocol for $^{45}Ca^{2+}$ influx was identical to that described for the study of the sorbitol space of the guinea-pig ileum, except that the radioactive isotope added after the 20-min control or drug incubation period was [^{45}Ca]Cl_2 (0.05 μCi/ml, final concentration). Tissues were incubated for periods of 5, 10, 20, 30, 45 and 120 minutes. The results for all time points were initially expressed in terms of total $^{45}Ca^{2+}$ space according to the above equation and were then converted to cellular Ca^{2+} influx (which included superficially bound Ca^{2+} and intracellular Ca^{2+}) by the following equation:

$$(\text{total } ^{45}Ca^{2+} \text{ space (ml/kg)} - [^3H]\text{-sorbitol space (ml/kg)}) \times \frac{1.35 \text{ mmol } Ca^{2+}}{1000 \text{ ml}} = \frac{\text{mmol } Ca^{2+}}{\text{kg (wet wt) tissue}}$$

For the study of Ca^{2+} efflux, the guinea-pig ileum was opened along its longitudinal axis and two pieces, each approximately 1 cm in length, were cut. Each piece was tied at both ends and mounted with approximately 1 g of tension between two hooks on a support. The two supports were then placed in two test tubes containing 10 ml of Tyrode solution which was maintained at 37° C throughout the experiment. The Tyrode solutions were oxygenated with 95% O_2 and 5% CO_2 throughout the experiment. After an initial equilibration period of 30 min, the tissues on the supports were transferred to two test tubes containing 10 ml of $^{45}Ca^{2+}$-Tyrode solution (2.0 μCi/ml) and a 2 h loading period was begun. The end of the 2 h loading period was designated as zero time and the two tissues on the supports were then simultaneously moved to two 15 ml centrifuge tubes. One tube (control) contained 5 ml of Tyrode solution and the other tube (experimental) contained 5 ml of Tyrode solution plus TMB-8 (65 μM). After 5 min, the mounted tissues were again moved to two other efflux tubes containing fresh 5 ml solutions of the identical respective compositions. Subsequent transfers of the mounted tissues occurred at 10, 20, 30, 45, 60, 90 and 120 minutes. At 120 min, the two pieces of ileum were removed from the mounts, blotted once and weighed. They were then placed in scintillation vials and prepared for scintillation counting as described in the preceding section. After thorough mixing of the contents of each efflux tube, a 0.5 ml sample was removed and added to a scintillation vial containing 10 ml of scintillation solution Similarly prepared for counting were 0.02 ml samples (in duplicate) taken from the two $^{45}Ca^{2+}$-Tyrode loading solutions. Identical samples were added to scintillation solution plus previously solubilized tissues to correct for tissue quenching. The samples were counted in a Beckman Scintillation System. From the $^{45}Ca^{2+}$ content of the tissue at 120 min and from the total $^{45}Ca^{2+}$ released during the selected intervals over the 2 h efflux period, the $^{45}Ca^{2+}$ remaining in the tissue during the efflux period was calculated. The results were expressed as the percent of $^{45}Ca^{2+}$ remaining in the tissue at the selected times, taking the tissue $^{45}Ca^{2+}$ content at zero time as 100% and plotted as desaturation curves.

Skeletal muscle sarcoplasmic reticulum preparation

New Zealand white rabbits, weighing 1 0-1.5 kg, were stunned by a blow on the head and decapitated. The two gastrocnemius muscles were isolated and removed. The sarcoplasmic reticulum of the rabbit gastrocnemius muscle was prepared according to the procedure of Ogawa, Harigaya & Ebashi (1971). From the final sarcoplasmic reticulum suspension 0.075 ml samples were taken to study $^{45}Ca^{2+}$ uptake and release. Pilot studies indicated that a 0.075 ml sample of this preparation would take up approximately 50% of the $^{45}Ca^{2+}$ contained in the incubation medium. Fresh reticulum fractions not older than 3 h were used for each experiment.

Ca^{2+} uptake into sarcoplasmic reticulum. The experimental protocol for Ca^{2+} uptake studies consisted of a 5 min preincubation period at 25° C of 0.075 ml of the final reticulum suspension with either 0.1 ml of distilled water (control) or 0.1 ml of distilled water solutions of TMB-8 (5 μM or 50 μM, final concentrations) in a 3 ml reaction mixture containing tris-maleate, 20 mM, KCl 100 mM; $MgCl_2$, 10 mM; $CaCl_2$, 0.01 mM and [^{45}Ca]Cl_2, 0.01 μCi/ml. The pH of the solution was 6.8. After this preincubation period, 0.1 ml of distilled water (control) or ATP (2.0 mM, final concentration) was added to initiate active Ca^{2+} uptake into the sarcoplasmic reticulum. After 2 min of uptake, 0.1 ml of distilled water was added. This step was included to provide appropriate controls for Ca^{2+} release experiments described below. One min after this addition, a

1.0 ml aliquot of the reaction mixture was rapidly passed through a millipore filter apparatus (0.22 μm pore size) which separated the reticulum and its associated Ca^{2+} from the free Ca^{2+} in solution. A 0.3 ml volume of the reticulum-free filtrate was placed in a counting vial containing 5 ml of Aquasol and counted in a Beckman Scintillation System. All control and experimental samples were run in duplicate. Initial observations confirmed the findings of other investigators (Scarpa, Baldassare & Inesi, 1972) that steady-state levels of Ca^{2+} uptake by skeletal muscle sarcoplasmic reticulum preparations are reached within 1 min at 25° C. A 3 min loading period was selected for Ca^{2+} uptake studies for the sake of experimental convenience.

Ca^{2+} release from sarcoplasmic reticulum. The experimental protocol of studies of Ca^{2+} release by caffeine consisted of a 5 min preincubation period identical to that described in the preceding section. After the preincubation period, ATP (2.0 mM, final concentration) was added to initiate active Ca^{2+} uptake into the sarcoplasmic reticulum. After 2 min, caffeine (20 mM, final concentration) was added. One min after this addition, a 1.0 ml aliquot of the reaction mixture was filtered as described in the preceding section and the subsequent steps also described in the preceding section were followed.

The amount of ^{45}Ca^{2+} taken up into the reticulum under all experimental conditions was calculated by subtracting the amount of free ^{45}Ca^{2+} from the total amount of free ^{45}Ca^{2+} in control experiments where distilled water was added in place of ATP. The results were converted into nmol of Ca^{2+}/g (wet wt) of muscle by appropriate conversions based on ct min^{-1} mol^{-1} Ca^{2+} in the unfiltered incubation medium.

Statistical analysis

All results were expressed as the mean ± s.e. mean. The significance of differences between two values was tested with Student's *t*-test and among three or more values with analysis of variance (Goldstein, 1964).

Results

Effect of TMB-8 on drug responses in the rabbit aortic strip

Table 1 shows the effects of TMB-8 (50 μM) on equivalent responses of the rabbit aortic strip to KCl and noradrenaline. It is important to note that responses to both KCl and noradrenaline were significantly inhibited by TMB-8 under the conditions of these experiments.

Table 1 The effects of TMB-8 on responses of rabbit aortic strips to equipotent doses of KCl and noradrenaline

	Response (g tension)		
Drug	*Control*	*TMB-8 (50 μM)*	*% Inhibition*
KCl, 90 mg	0.86 ± 0.09	0.18 ± 0.03*	78.5 ± 4.1
Noradrenaline, 0.1 μg	0.80 ± 0.10	0.43 ± 0.05*	46.4 ± 5.2

Values are mean with s.e. mean of 5 experiments.
* Significantly different from control responses at $P < 0.01$ as determined by Student's *t* test (Goldstein, 1964).

Table 2 The effects of TMB-8 on the [^{3}H]-sorbitol space of the guinea-pig ileum

Incubation time (min)	*[^{3}H]-sorbitol space (ml/kg tissue)*	
	Control	*TMB-8 (65 μM)*
5	110.8 ± 8.2	127.0 ± 24.9
10	159.6 ± 24.2	146.7 ± 7.9
20	168.2 ± 10.6	187.7 ± 24.3
30	192.3 ± 17.5	197.9 ± 17.1
45	203.7 ± 18.1	220.2 ± 13.2
120	319.6 ± 19.5	344.7 ± 21.3

Values are mean with s.e. mean of 5 experiments.

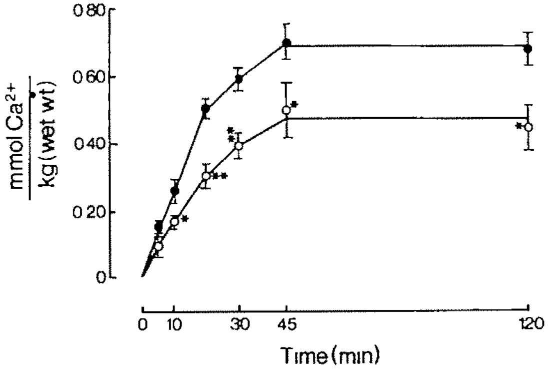

Figure 1 Effects of TMB-8 on resting cellular Ca^{2+} influx into the guinea-pig ileum preparation. (●) Control influx; (○) influx in the presence of TMB-8 65 μM. Each point is the mean of 5 values and vertical bars indicate s.e. mean.
* $P < 0.05$ compared with control value; ** $P < 0.01$.

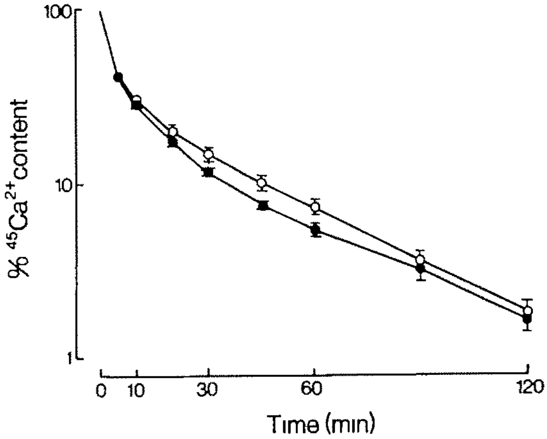

Figure 2 Effects of TMB-8 on the resting $^{45}Ca^{2+}$ efflux in the guinea-pig ileum preparation. (●) Control efflux of $^{45}Ca^{2+}$; (○) efflux of $^{45}Ca^{2+}$ in the presence of TMB-8 65 μM. Each point is the mean of 5 values and the vertical bars indicate s.e. mean.

Effect of TMB-8 on the extracellular space of the guinea-pig ileum

TMB-8 at a concentration of 65 μM had no effect on the extracellular space of the guinea-pig ileum as measured with [^{3}H]-sorbitol during the 2 h incubation period (Table 2).

Effect of TMB-8 on the resting cellular Ca^{2+} influx and efflux in the guinea-pig ileum

Figure 1 illustrates the effect of TMB-8 on the resting cellular Ca^{2+} influx in the guinea-pig ileum. TMB-8 (65 μM) significantly inhibited the influx of Ca^{2+} at 10, 20, 30, 45 and 120 minutes.

Figure 2 illustrates the effect of TMB-8 on the resting $^{45}Ca^{2+}$ efflux from the guinea-pig ileum. TMB-8 (65 μM) produced a small but significant reduction ($P < 0.05$) of the percentage of $^{45}Ca^{2+}$ lost from this tissue after 30, 45 and 60 min washing.

Table 3 The effects of TMB-8 on Ca^{2+} uptake into the sarcoplasmic reticulum of rabbit gastrocnemius muscle

Treatment	*Ca^{2+} uptake (nmol/g muscle)*
None	16.38 ± 1.62
TMB-8 (5 μM)	15.82 ± 0.76
TMB-8 (50 μM)	16.30 ± 0.98

Values are mean with s.e. mean of 5 experiments.

Effect of TMB-8 on $^{45}Ca^{2+}$ uptake and release in skeletal muscle sarcoplasmic reticulum

Table 3 shows that TMB-8 at concentrations of 5 μM and 50 μM had no effect on active $^{45}Ca^{2+}$ uptake into the sarcoplasmic reticulum of rabbit skeletal muscle. Table 4 indicates that TMB-8 at a concentration of 5 μM significantly decreased the quantity of $^{45}Ca^{2+}$ released from the sarcoplasmic reticulum of rabbit skeletal muscle by 20 mM caffeine.

Table 4 The effects of TMB-8 on caffeine-induced release of Ca^{2+} from the sarcoplasmic reticulum of rabbit gastrocnemius muscle

Treatment	*Ca^{2+} content (nmol/g muscle)*
None	16.38 ± 1.62^{a*}
Caffeine (20 mM)	11.34 ± 0.49^{b}
Caffeine (20 mM) plus TMB-8 (5 μM)	13.46 ± 0.56^{c}

Values are mean with s.e. mean of 5 experiments.
* The presence of a different letter in the superscripts of the means ± s.e. mean indicates a significant difference at the $P < 0.05$ level among these values as determined by analysis of variance (Goldstein, 1964).

Discussion

It has been shown that responses of the rabbit aorta to KCl are dependent upon Ca^{2+} influx into the smooth muscle cell, while phasic responses of vascular smooth muscle to noradrenaline are independent of extracellular Ca^{2+} concentrations and are due to the direct release of Ca^{2+} from intracellular stores by this agent (Hudgins & Weiss, 1968; Godfraind & Kaba, 1969). The inhibition of KCl and phasic noradrenaline responses of the rabbit aorta produced by TMB-8 in the present study suggests that this agent may (1) act at the membrane to inhibit the influx of extracellular Ca^{2+}; (2) act at intracellular Ca^{2+} storage sites to block the release of Ca^{2+} from these stores; or (3) act at both of these sites to reduce the availability of Ca^{2+} to the contractile apparatus

The inhibition of resting cellular $^{45}Ca^{2+}$ influx in the guinea-pig ileum by TMB-8 suggests that TMB-8 does have a significant inhibitory effect on the movement of Ca^{2+} into the smooth muscle cell. The decrease in the percentage of $^{45}Ca^{2+}$ which effluxes from the guinea-pig ileum suggests that TMB-8 might also slow the release of Ca^{2+} from cellular stores in smooth muscle. However, it is not possible from this study to state at which site or sites of Ca^{2+} storage in smooth muscle TMB-8 exerts this inhibition.

In the sarcoplasmic reticulum preparation of skeletal muscle, TMB-8 caused potent inhibition of caffeine-induced Ca^{2+} release at a concentration (5 μM) that was well within the range of doses of TMB-8 required to block responses of skeletal muscle *in vitro* (1.8 μM to 470 μM) (Malagodi & Chiou, 1974b). However, TMB-8 had no effect on the uptake of $^{45}Ca^{2+}$ by this preparation, indicating that TMB-8 did not inhibit Ca^{2+} availability by enhancing Ca^{2+} uptake by the sarcoplasmic reticulum.

It is possible that TMB-8 may have produced all of its inhibitory effects on smooth and skeletal muscle by stabilizing Ca^{2+} binding to functionally important cellular Ca^{2+} sequestering sites. In so doing, TMB-8 could antagonize Ca^{2+} release from cellular Ca^{2+} sequestering sites regardless of whether it is elicited via depolarization by KCl or direct release by caffeine or noradrenaline. The inhibition of $^{45}Ca^{2+}$ influx into the guinea-pig ileum may be the result of an inhibition of the displacement of unlabelled Ca^{2+} by labelled Ca^{2+} at sarcolemmal and/or microsomal sites, an effect that may have been due to a TMB-8-induced stabilization of Ca^{2+} binding. The decrease in the percentage of effluxing $^{45}Ca^{2+}$ from the guinea-pig ileum and the inhibition of $^{45}Ca^{2+}$ release from the skeletal muscle sarcoplasmic reticulum preparation also support this proposed mechanism. An agent affecting such a late step in the common pathway of all contractile stimuli as the release of Ca^{2+} (Daniel, 1964) may prove to be a valuable tool in further studies of the basic mechanism of contraction and relaxation in muscles of all types.

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LIVER AND BRAIN TRYPTOPHAN METABOLISM FOLLOWING HYDROCORTISONE ADMINISTRATION TO RATS AND GERBILS

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1 Liver tryptophan pyrrolase activity is low in the mongolian gerbil (*Meriones unguiculatus*) and is not induced by hydrocortisone (5 mg/kg). In contrast, there is measurable activity in the rat liver and this is induced by hydrocortisone. *In vivo* measurements confirmed the absence of induction in gerbils but suggested that they were able to metabolize tryptophan However, no detectable pyrrolase activity was found in any other tissues either before or after hydrocortisone.

2 In agreement with previous observations hydrocortisone decreased rat brain 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) 6 h after administration. Brain tryptophan concentrations were also decreased at this time. In contrast, hydrocortisone did not alter gerbil brain 5-HT, 5-HIAA or tryptophan. α-Methyltryptophan activated hepatic tryptophan pyrrolase and decreased brain 5-HT and 5-HIAA in both animals.

3 Results suggest that the decrease in rat brain 5-HT and 5-HIAA following hydrocortisone may be associated with the rise in liver tryptophan pyrrolase and that the brain amine changes are mediated through the decrease in brain tryptophan concentration.

Introduction

Intraperitoneal injection of hydrocortisone (5 mg/kg) into rats decreases the concentrations of 5-hydroxytryptamine (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) in the brain, suggesting decreased 5-HT synthesis (Curzon & Green, 1968; Yuwiler, Wetterberg & Geller, 1971). Corticosterone and the synthetic glucocorticoid betamethasone produce comparable changes (Scapagnini, Preziosi & DeSchaepdryver, 1969). The change in brain 5-HT metabolism may be a consequence of altered peripheral tryptophan metabolism, because hepatic tryptophan pyrrolase activity is induced by corticosteroids (Knox & Auerbach, 1955), and injection of the pyrrolase inhibitor, allopurinol, prevents the brain amine changes (Green & Curzon, 1968). α-Methyltryptophan also increases pyrrolase activity and decreases rat brain 5-HT and 5-HIAA (Green & Curzon, 1968; Sourkes, Missala & Oravec, 1970).

It has recently been reported that pyrrolase activity is low in the mongolian gerbil (*Meriones unguiculatus*), and that it is not induced by hydrocortisone (Baughman & Franz, 1971). We have now investigated the differences in hepatic tryptophan metabolism in rats and gerbils, both before and after hydrocortisone. The effect of hydrocortisone on 5-HT metabolism in both rat and gerbil brain has also been examined.

Methods

Adult male gerbils 50-90 g (MRC Laboratory Animals Centre, Carshalton, Surrey, and Canadian Breeding Farm and Laboratory, St Constant, Quebec, Canada) and male Sprague-Dawley rats 140-160 g (Carworth-Europe, Alconbury, Huntingdon and Canadian Breeding Farm and Laboratory) were used. They were fed an *ad libitum* diet of small animal 41B pellets and tap water. They were killed by decapitation at the same time of day (14 h 30 min-16 h 30 min) to minimize the effects of possible diurnal variations of pyrrolase or 5-HT. The following drugs were used: hydrocortisone sodium succinate (Solu-Cortef, Upjohn Co.), DL-α-methyltryptophan (Upjohn Co., Kalamazoo, Mo., U.S.A.), L-kynurenine sulphate (Sigma Chemical Co) and DL-[ring-2-^{14}C]-tryptophan

(2 mCi/mmol; I.C.N. Research Products, Montreal, Quebec, Canada). All drugs were dissolved in 0.9% w/v NaCl solution (saline) and injected intraperitoneally. The hydrocortisone dose is quoted as free steroid. Control animals were injected with isotonic saline.

Analytical methods

Pyrrolase activity was measured *in vitro* in supernatants (16,000 *g* for 1 h) from 25% homogenates after preincubation of the soluble liver fraction with methaemoglobin, tryptophan and freshly neutralized ascorbic acid (Knox, Piras & Tokuyama, 1966), or thermal activation, when the soluble liver fraction was incubated at 55°C for 5 min, chilled, centrifuged and the supernatant used (Schutz & Feigelson, 1972). Measurement of pyrrolase activity in liver homogenates was essentially by the method of Knox & Auerbach (1955) in which whole liver homogenates are incubated in the presence of 0.003 M tryptophan, except that 2×10^{-6}M haematin was added to the reaction mixture (Kevitz & Wagner, 1965). Pyrrolase activity *in vivo* was determined by the method of Madras & Sourkes (1968) as modified by Young, Oravec & Sourkes (1974). Rats were injected with hydrocortisone (50 mg/kg) 5 h before DL-[ring-2-^{14}C]-tryptophan (2.5 μmol/kg). The animals were then placed in glass metabolism cages, without food or water, through which CO_2-free air was drawn. Labelled CO_2 was trapped by bubbling the expired gas through ethyleneglycol monomethyl ether-ethanolomine (2 : 1). Aliquots of this solution were removed, mixed with scintillant and counted by scintillation spectrometry.

Brain 5-HT and 5-HIAA were measured by the method of Curzon & Green (1970) and brain tryptophan by that of Denkla & Dewey (1967).

Results

In vitro *activity of tryptophan pyrrolase in rat and gerbil liver after hydrocortisone*

Rat liver pyrrolase activity in whole liver homogenates was essentially the same as previously reported (Green & Curzon, 1968) and the rise in activity 3 h after hydrocortisone (5 mg/kg) was also as previously seen (Table 1). However, in the gerbil, hepatic pyrrolase activity was only just detectable (Table 1) and the value is probably not significant (see discussion section). Furthermore, hydrocortisone administration did not cause a statistically significant increase in pyrrolase activity 3 h after administration. Since enzyme induction in the gerbil might have occurred at a different time from that in the rat, pyrrolase activity was also measured in groups of 4 gerbils at 2, 4, 5 and 6 h after injection. However, no significant increase in activity was found at any time after hydrocortisone administration.

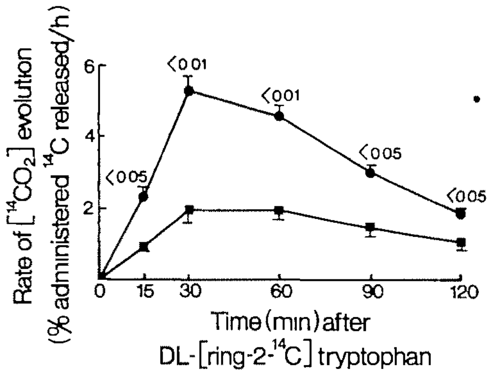

Figure 1 Effect of hydrocortisone (50 mg/kg) on [$^{14}CO_2$] release in the rat following injection of DL-[ring-2-^{14}C] tryptophan (2.5 μmol/kg). Each point is mean of 6 animals Vertical bars indicate s.e. mean. Rats were injected with hydrocortisone 50 mg/kg (●) or saline (■) 4 h before injection of DL-[ring-2-^{14}C] tryptophan (2.5 μmol/kg). [$^{14}CO_2$] evolution was measured as described in methods section. Significance compared to control shown for each point

The low activity in gerbil liver might have been due to inadequacies in the method used. Therefore we tried, but failed, to detect significant activity in gerbil liver supernatants after preincubation with methaemoglobin according to Knox *et al.* (1966) and after thermal activation, by the method of Schutz & Feigelson (1972). Furthermore, addition of an equal volume of gerbil homogenate to a rat liver homogenate had no effect on either conjugation or activity of the rat pyrrolase as measured by the method of Knox *et al.* (1966).

In vivo *activity of tryptophan pyrrolase in rat and gerbil liver after hydrocortisone*

The rate of tryptophan degradation was measured in both rats and gerbils by the *in vivo* techniques of Madras & Sourkes (1968). Rats showed an increase in [$^{14}CO_2$] evolution following hydrocortisone (Figure 1). In agreement with the *in vitro* results, there was no statistically significant increase in [$^{14}CO_2$] excretion after hydrocortisone in the gerbil even at a dose of 50 mg/kg. However, the radioactive tryptophan was metabolized by the gerbil suggesting pyrrolase activity *in vivo* (Figure 2).

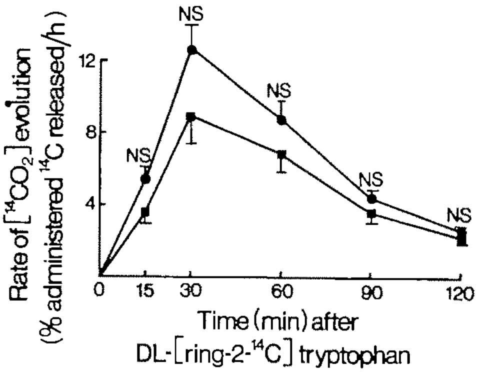

Figure 2 Effect of hydrocortisone (50 mg/kg) on [$^{14}CO_2$] release in the gerbil following injection of DL-[ring-2-^{14}C] tryptophan (2.5 μmol/kg). Each point shows mean of 9 animals. Vertical bars indicate s.e. mean. Gerbils were injected with hydrocortisone 50 mg/kg (●) or saline (■) 4 h before injection of DL-[ring-2-^{14}C] tryptophan (2.5 μmol/kg). [$^{14}CO_2$] evolution was measured as described in methods section. NS—not significantly different from saline-injected control.

Examination of other gerbil tissues for pyrrolase activity

To determine whether the radioactive tryptophan was being degraded by tissues other than the liver, the *in vitro* assay of Knox *et al.* (1966) was used to see whether there was detectable pyrrolase activity in other tissues. No significant pyrrolase activity was found in gerbil intestine, kidney, spleen, heart or brain either before or after hydrocortisone treatment.

Effect of hydrocortisone on gerbil and rat brain 5-hydroxytryptamine, 5-hydroxyindoleacetic acid and tryptophan

In agreement with previous observations (Green & Curzon, 1968), rat brain 5-HT and 5-HIAA decreased 6 h after hydrocortisone (5 mg/kg) (Table 1). We also found, in agreement with Curzon & Knott (personal communication), that brain tryptophan concentrations were decreased at this time (Table 1).

Table 1 Effect of hydrocortisone and α-methyltryptophan on activity of gerbil and rat liver tryptophan pyrrolase and brain tryptophan, 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) concentrations

Injected (i.p.)	*Pyrrolase activity (μmol kynurenine h^{-1} g^{-1} liver (dry wt))*	*Brain tryptophan (μg/g brain (wet wt))*	*Brain 5-HT (μg/g brain (wet wt))*	*Brain 5-HIAA (μg/g brain (wet wt))*
Gerbils				
Saline	1.86 ± 0.86 (6)	6.07 ± 1.04 (6)	0.57 ± 0.03 (9)	0 44 ± 0.02 (9)
Hydrocortisone (5 mg/kg)	3.32 ± 0.54 (6)	6.97 ± 0 20 (6)	0.55 ± 0.04 (12)	0.45 ± 0.05 (12)
DL-α-methyl tryptophan (25 mg/kg)	13.51 ± 0.99* (6)	ND	0.50 ± 0.01** (6)	0.30 ± 0.02* (6)
Kynurenine (5 mg/kg)	ND	5.80 ± 1.06 (6)	0.56 ± 0.03 (4)	0.44 ± 0.02 (4)
Rats				
Saline	6.02 ± 0.85 (6)	6.81 ± 0.24 (10)	0.52 ± 0.02 (10)	0.36 ± 0.02 (10)
Hydrocortisone (5 mg/kg)	20.02 ± 3.42* (6)	4.03 ± 0.27** (6)	0.41 ± 0.02* (6)	0.23 ± 0.03* (6)
DL-α-methyl tryptophan (25 mg/kg)	44.46 ± 1.74* (4)	ND	0.44 ± 0.02** (6)	0.23 ± 0.03* (6)
Kynurenine (5 mg/kg)	ND	3.45 ± 0.68** (6)	0.41 ± 0.03* (6)	0.25 ± 0.03* (6)

Pyrrolase activity measured 3 h after injection. 5-HT, 5-HIAA and tryptophan measured 6 h after injection, except kynurenine where brain tryptophan results are shown 1 h after injection and 5-HT and 5-HIAA 2 h after injection.

* Different from saline treated $P < 0.001$. ** Different from saline-treated, $P < 0.01$. ND Not determined.

In contrast, hydrocortisone injection at the same dose elicited in the gerbil neither a change in 5-HT and 5-HIAA, nor a decrease of brain tryptophan (Table 1).

Effect of α-methyltryptophan on liver pyrrolase, brain 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in rats and gerbils

Injection of α-methyltryptophan into rats activates tryptophan pyrrolase (Sourkes & Townsend, 1955). The increase in the enzyme activity following tryptophan and α-methyltryptophan occurs in adrenalectomized rats (Civen & Knox, 1960) and is thus not mediated by glucocorticoids However, unlike tryptophan, α-methyltryptophan is not a substrate for the enzyme (Sourkes & Townsend, 1955). Baughman & Franz (1971) reported that gerbil pyrrolase activity was increased by injection of the substrate tryptophan. The effect of the analogue α-methyltryptophan was therefore examined. α-Methyltryptophan (25 mg/kg) increased pyrrolase activity as measured in whole liver homogenates from both rats and gerbils. It also produced a decrease in brain 5-HT and 5-HIAA in both rodents (Table 1).

Effect of L-kynurenine on rat and gerbil brain tryptophan, 5-hydroxytryptamine and 5-hydroxyindoleacetic acid

During an investigation into the possible mechanism of the proposed pyrrolase-5-HT association it was found that the product of pyrrolase activity, L-kynurenine, injected in a dose of 5 mg/kg, decreased brain 5-HT concentrations 2 h after injection (Green & Curzon, 1970). This was confirmed in the current study (Table 1). This compound did not influence gerbil brain 5-HT and 5-HIAA concentrations 2 h after injection (Table 1), nor was any change noted 1 h or 3 h after injection.

Although brain tryptophan concentrations were not decreased in the rat or gerbil brain 2 h after kynurenine injection (rat. 6.70 ± 0.56 μg/g ($n = 6$), gerbil: 6.13 ± 0.76 μg/g ($n = 6$)), a decrease in concentration was found in the rat brain 1 h after injection. However, concentrations were unaltered in the gerbil (Table 1).

Discussion

Baughman & Franz (1971) found no significant pyrrolase activity in gerbil liver *in vitro*. In the present study measurable values were found. However, the results of Baughman & Franz (1971) are expressed as μmol kynurenine formed h^{-1} g^{-1} liver (wet wt), whereas those of this study are expressed as μmol kynurenine formed h^{-1} g^{-1} liver (dry wt). If our results are divided by the wet wt/dry wt ratio for liver of 3.45 (Woods & Krebs, 1971) this gives a value for pyrrolase activity of 0.5 μmol kynurenine formed h^{-1} g^{-1} liver (wet wt) which is in excellent agreement with the results of Baughman & Franz (1971). Franz & Knox (1967) have discussed the criteria for definite enzymic activity and concluded that anything less than 1.0 μmol kynurenine formed h^{-1} g^{-1} liver (wet wt) is not indicative of true enzyme activity, due to the limitations of the spectrophotometric method.

In rat liver, tryptophan pyrrolase exists in at least three forms, the inactive apoenzyme (Greengard & Feigelson, 1962) and the oxidized and reduced holoenzyme (Tanaka & Knox, 1959; Tokuyama & Knox, 1964). While addition of tryptophan and the co-factor haematin activates the enzyme in whole homogenates this only occurs after a time lag (Knox & Ogata, 1965). We therefore also attempted to measure pyrrolase activity in cell supernatants following either preincubation with methaemoglobin (Knox *et al.*, 1966) or thermal activation (Schutz & Feigelson, 1972), both techniques being designed to ensure that all enzyme present is converted to the active reduced holoenzyme before assay. Even with these assay systems no significant enzyme activity was found in the gerbil. It must be remembered however that none of these assay methods can demonstrate the absence of enzyme activity, merely that its activity is below an ascribed amount.

No detectable enzyme activity was found in any gerbil tissues examined. Furthermore, gerbil liver homogenates had no effect on either conjugation or activity of rat liver homogenates, suggesting the absence of an endogenous inhibitor. Clearly this cannot be ruled out since a gerbil liver inhibitor would not necessarily inhibit rat pyrrolase. Nevertheless, this absence of enzyme activity when measured *in vitro* made the *in vivo* results surprising. Results clearly suggested that the gerbils degraded tryptophan more rapidly than rats, indicating pyrrolase activity.

There are two main possibilities to account for this discrepancy. The first is that tryptophan is metabolized in the gerbil by a different degradative route not involving pyrrolase. However, if this is the case it is surprising that formamidase activity in the liver is normal (Baughman & Franz, 1971). The second and more likely situation is that the *in vitro* assay is not reflecting the *in vivo* condition, a situation that has been observed with other enzyme systems (see review by Youdim & Woods, 1975).

Although the discrepancy between the *in vivo* and *in vitro* assays is not explained, they are in agreement on the lack of effect of hydrocortisone in the gerbil, unlike the rat. The *in vitro* assay shows no significant increase in enzymic activity and the *in vivo* assay demonstrates that there is no increase in the rate of tryptophan degradation after hydrocortisone It is unlikely that the lack of induction is due to a more rapid turnover, or excretion, of hydrocortisone in gerbils, since doses as high as 500 mg/kg have no effect on pyrrolase (Baughman & Franz, 1971).

In confirmation of previous findings, injection of hydrocortisone (5 mg/kg) induced rat hepatic pyrrolase activity and decreased brain 5-HT and 5-HIAA. In contrast, the same dose of hydrocortisone, which in gerbils does not induce pyrrolase, also has no effect on brain 5-HT metabolism in these animals. This suggests an association between pyrrolase activity and brain 5-HT metabolism and is consistent with the observation that the pyrrolase rise and the brain 5-hydroxyindole decrease following hydrocortisone both diminish with increasing age (Green & Curzon, 1975). The decrease in rat brain 5-HT concentrations in younger rats following hydrocortisone presumably results from the fall in brain tryptophan concentration (Table 1). It seems reasonable to assume that this decrease of brain tryptophan is due to the increase in tryptophan pyrrolase activity, especially as the gerbil shows neither an enzyme rise nor a brain tryptophan decrease. In a previous communication we were unable to find a significant fall in brain tryptophan (Green, Joseph & Curzon, 1970). However, measurements were then made 3 h after hydrocortisone, not at 6 h, which is when the major 5-HT decrease has occurred (Curzon & Green, 1968).

Activation of gerbil pyrrolase after α-methyltryptophan is of the same magnitude as that observed in the rat (7-fold) and is consistent with the finding that gerbil pyrrolase activity can be increased by substrate (Baughman & Franz, 1971). Furthermore, this compound caused a decrease in gerbil brain 5-HT and 5-HIAA as it does in rats (Table 1; Green & Curzon, 1968; Sourkes *et al.*, 1970). The larger decrease of 5-HIAA than 5-HT has been reported previously (Sourkes *et al.*, 1970; Curzon & Green, 1971) and is due to the formation of α-methyl-5-HT which produces falsely high values (Roberge, Missala & Sourkes, 1971, 1972).

It is probable that α-methyltryptophan decreases brain 5-HT because of the increase of pyrrolase activity since there is a large decrease of both plasma and brain tryptophan following administration of this compound when pyrrolase activity is high (Sourkes *et al.*, 1970)

In the rat, kynurenine injection produced a significant decrease in brain 5-HT and 5-HIAA concentrations This is probably because it competes with tryptophan for uptake into the brain, since kynurenine decreases accumulation of labelled tryptophan in brain slices (Green & Curzon, 1970; Kiely & Sourkes, 1972) and decreases rat brain tryptophan 1 h following injection. Thus the finding that this compound had no effect on brain tryptophan concentrations and did not decrease brain 5-HT or 5-HIAA concentrations in the gerbil again indicates differences in tryptophan metabolism between these two rodents. It is possible that accumulation of tryptophan by the gerbil brain is by a different mechanism from that in the rat. However, Leklem, Woodford & Brown (1969), working with the cat (another mammal in which pyrrolase is not subject to hormonal induction), recovered very little of an injected dose of kynurenine in the urine. They suggested that this was because the compound was rapidly conjugated and therefore was undetectable with their assay methods. This might also be true in the gerbil, since the conjugated compound would presumably not affect cerebral amino acid transport.

While it is not clear what the physiological reasons are for the differences in tryptophan metabolism in rat and gerbil our results add weight to the hypothesis that increased pyrrolase activity is associated with a decrease in brain 5-HT synthesis and suggest that this decrease in 5-HT synthesis is the result of a decrease in brain tryptophan concentrations.

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THE EFFECTS OF BURIMAMIDE AND METIAMIDE ON BASAL GASTRIC FUNCTION IN THE CAT

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1 Burimamide injected intravenously in the anaesthetized or conscious cat produced significant increases in gastric acid secretion. in the anaesthetized cat it produced increased gastric mucosal blood flow

2 Metiamide, in doses which inhibited pentagastrin-stimulated acid secretion, produced no increase in gastric acid secretion in conscious animals, or gastric acid secretion or gastric mucosal blood flow in the anaesthetized cat.

3 Metiamide did not influence the amount of acid which diffused out of the stomach when instilled at pH values between 1.5 and 6.0.

4 The possible mode of action of burimamide in increasing basal gastric secretion is discussed.

Introduction

Burimamide (N-methyl-*N'*-(4-(4(5)-imidazolyl)-butyl)thiourea) has been reported as a competitive inhibitor of histamine H_2-sites based on data obtained on guinea-pig isolated atrium and rat isolated uterine horn and *in vivo* experiments on gastric acid secretion in rat, cat, dog and man (Black, Duncan, Durant, Ganellin & Parsons, 1972; Wyllie, Hesselbo & Black, 1972). A later compound, metiamide (N-methyl-*N'*-(2[(5-methyl imidazol-4-yl)methylthio]-ethyl)thiourea) has also been shown to antagonize competitively the action of histamine on atrial and uterine receptors and to be approximately ten times more powerful in this respect than burimamide and to be approximately five times more potent in reducing histamine- or pentagastrin-stimulated acid secretion *in vivo* (Black, Duncan, Emmett, Ganellin, Hesselbo, Parsons & Wyllie, 1973).

This paper describes an investigation into the effects of burimamide and metiamide on basal gastric acid secretion and blood flow in the anaesthetized cat, and on acid secretion in the conscious cat. We have also studied back diffusion of H^+ from the stomach in anaesthetized cats.

Methods

All animals were starved for 36 h before experiments but allowed free access to water.

Anaesthetized cats

Anaesthesia was induced with ether and maintained by a single intravenous injection of chloralose (80 mg/kg) The vagus nerves were sectioned in the neck and the splanchnic nerves cut extraperitoneally. The stomach was intubated with a wide bore catheter via an incision in the cervical portion of the oesophagus and the pylorus occluded with a tape ligature. Arterial blood samples were taken through a catheter placed in a femoral artery; this catheter also served to monitor arterial blood pressure.

Routinely 50 ml of a 5 : 1, v/v mixture of 300 mosmol/kg solutions of mannitol and glycine, adjusted to pH 3.4 by addition of HCl, was instilled into the stomach at the beginning of each 15 min collection period, drained between 14 and 15 min and replaced by a further 50 ml. Acid content was determined by electrometric titration of a 25 ml sample to pH 7.0 with 0.02N NaOH. Samples of gastric juice washout fluid were stored for measurement of mucosal blood flow by the technique of Harper, Reed & Smy (1968), described for use in the anaesthetized cat.

Amidopyrine concentrations in gastric washings and arterial plasma were determined by the method of Brodie & Axelrod (1950). Burimamide or metiamide was given by a single intravenous injection (2 mg/kg) during basal secretion of acid

In a series of three experiments the effect of metiamide on the back diffusion of H^+ from the stomach was investigated. Fifty ml aliquots of mixtures of isosmotic (300 mosmol/kg) solutions of mannitol, glycine and HCl over the pH ranges 6.0, 4.0, 3 0, 2.5, 2 0 and 1 5 were instilled into the stomach. Loss of H^+ was determined by subtraction of the recovered acid, estimated by titration after 15 min in the stomach, from the known amount of acid instilled.

Conscious cats

These experiments were carried out on ten cats in which chronic gastric fistulae (Emås, 1960) had been prepared at least three months earlier. Animals were given no solid food for 36 h prior to experiments but were allowed milk up to 16 h before each experiment. all animals were allowed free access to water.

During experiments animals were supported in canvas slings and the gastric secretions collected in small, calibrated plastic receptacles attached to the cannulae. The collection vessels were replaced at 15 min intervals and the contents titrated to pH 7 0 with 0.05N NaOH to determine acid output Experiments were carried out in five groups·

(i) basal acid secretion.
(ii) effect of burimamide (2 mg/kg i.v.) on basal acid secretion.
(iii) acid response to pentagastrin (Peptavlon, ICI, 6 μg/kg s.c.).
(iv) effect of metiamide (4 mg/kg i.m.) on the acid response to pentagastrin.
(v) effect of metiamide (4 mg/kg i.m.) on basal acid secretion.

Results are expressed as means ± s.e. mean; significant difference between means was determined by Student's *t* test or Cochran's modification depending on analysis of variance.

Burimamide and metiamide were supplied by Smith, Kline & French.

Results

Anaesthetized cats

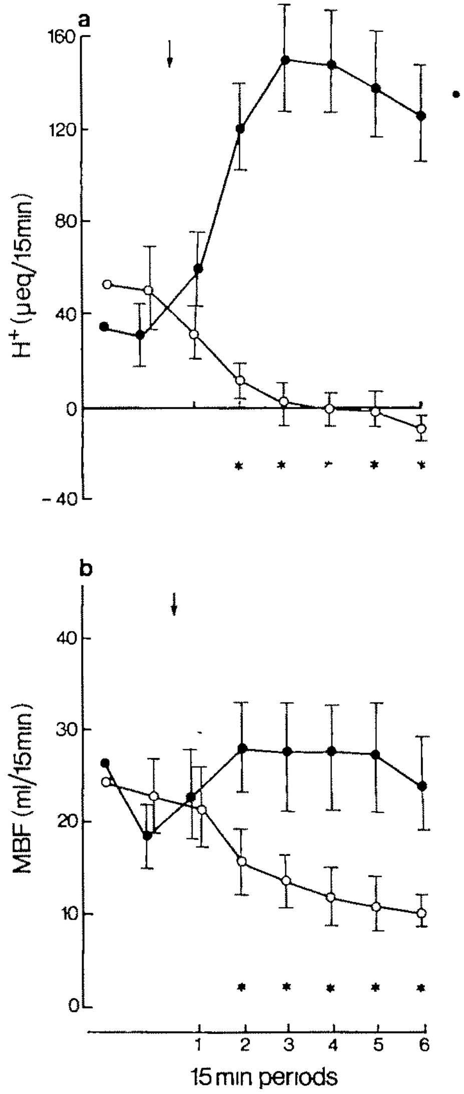

Figure 1 The effects of injection (at arrow) of burimamide (●) (2 mg/kg i.v.) and metiamide (○) (2 mg/kg i.v.) on (a) gastric acid secretion and (b) gastric mucosal blood flow (MBF) in eight anaesthetized cats. Results are expressed as mean; vertical lines show s.e. mean. Significant differences between means at the 5% level are indicated by asterisks.

In eight anaesthetized cats the injection of burimamide 2 mg/kg intravenously during basal acid secretion caused a five-fold increase of mean acid secretion and increased the mean resting mucosal blood flow (MBF) by 46% (Figure 1). In the period before injection of burimamide the mean basal acid secretion was 30.9 ± 14.8 μEq H^+/15 min and the mean resting MBF was 18.7 ± 3.7 ml/15 minutes. Peak acid secretion was seen during the third 15 min period after the injection of burimamide, 150.9 ± 24.3 μEq H^+/15 min (Figure 1a) and was accompanied by a MBF of 27.4 ± 5.4 ml/15 min (Figure 1b).

By contrast, in six other cats injection of

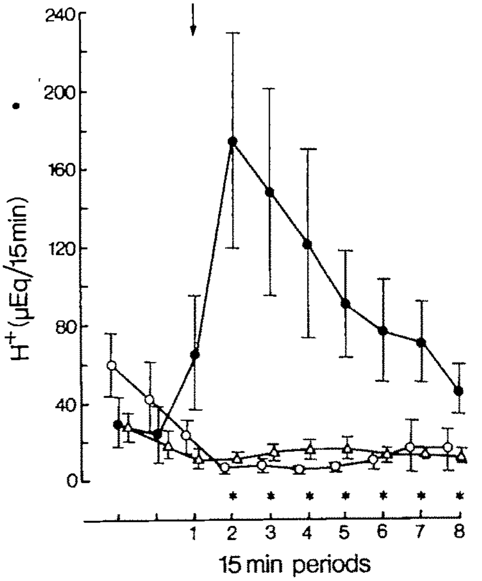

Figure 2 Results in conscious cats with gastric fistulae. The effects of injection (at arrow) of burimamide (●) (2 mg/kg i.v. in nine cats) and metiamide (○) (4 mg/kg i.m. in six cats) on gastric acid secretion compared with acid secretion in the basal state (△) in 10 cats. Results are expressed as mean; vertical lines show s.e. mean. Significant differences between burimamide-stimulated mean responses and basal secretion means are indicated by asterisks.

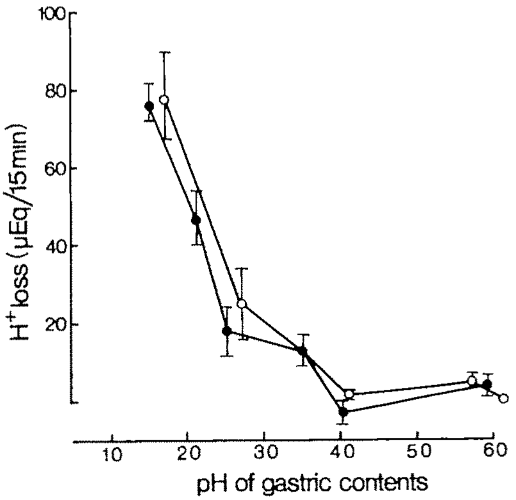

Figure 3 Comparison of H⁺ loss from the stomach in anaesthetized cats at various intragastric pH values under basal conditions (○) and during intravenous infusion of metiamide (●) 2 mg kg⁻¹h⁻¹, following intravenous injection of 2 mg/kg. The pH values of the instilled gastric fluid are shown on the abscissae, H⁺ loss (μEq/15 min) on the ordinates Results are shown as means at each pH value studied; vertical lines show s.e. mean There is no significant difference between corresponding means.

metiamide (2 mg/kg i.v.) during basal acid secretory conditions caused a decrease in acid output from 51.0 ± 18.4 μEq H^+/15 min to 0.8 ± 8.6 μEq H^+/15 min during the third 15 min period following the injection (Figure 1a). This reduction of acid output continued during the 1.5 h period studied. MBF also declined from 22.9 ± 4.3 ml/15 min before injection of metiamide, to 13.3 ± 2.7 ml/15 min in the third post injection period, and continued to decline over the period studied (Figure 1b).

The mean acid outputs and mean MBF values during the second and subsequent response following burimamide were significantly greater than those during the corresponding periods after metiamide ($P < 0.05$ in each case).

Conscious cats

Basal acid secretion in ten cats was small and declined slowly over the 2 h studied (Figure 2). Injection of burimamide (2 mg/kg i.v.) in nine cats produced an increase in acid secretion from 24.1 ± 15.0 μEq H^+/15 min during basal conditions to a peak 174.1 ± 56.4 μEq H^+/15 min 30 min after the injection (Figure 2). The mean acid outputs after injection of burimamide were significantly greater than those in control experiments ($P < 0.05$) with the exception of the first period after the injection (Figure 2).

Metiamide (4 mg/kg i.m.) was injected in six animals secreting acid under basal conditions. There was no significant change in acid secretion compared with the control experiments (Figure 2). Metiamide was injected intramuscularly because of its greater solubility and the dose, 4 mg/kg, was shown to be capable of reducing pentagastrin-stimulated acid secretion. In five animals, acid secretion was stimulated by a single subcutaneous injection of pentagastrin. The total acid secreted in the subsequent 2 h was measured and designated the 100% response in that animal. In the same animals the same dose of pentagastrin was accompanied by injections of metiamide (4 mg/kg i.m.) and the total acid output expressed as a per cent of the 100% response in that animal. The results of the two series were compared and showed a significant reduction of the mean of 64.4% ($P < 0.001$) when pentagastrin was injected with metiamide.

Back diffusion of acid out of the gastric lumen was estimated in three anaesthetized cats during infusion of metiamide, 2 mg kg^{-1} h^{-1}, following an intravenous injection of 2 mg/kg. The amounts of H^+ lost from the stomach over the pH range 6.0 to 1.5 were compared with those obtained under basal control conditions by Harper, Reed & Smy (1970). There was no significant increase of H^+ loss from the stomach under the influence of metiamide (Figure 3).

Discussion

Burimamide increased the acid secretion and MBF from basal levels in the anaesthetized cat (Figure 1). An almost identical increase in acid secretion was seen following burimamide in the conscious cat (Figure 2). MBF in the conscious animal was not measured.

It is well established that burimamide causes reduction of acid secretion stimulated by histamine or gastrin in many species. (International Symposium on Histamine H_2-receptor antagonists; Smith Kline & French (1973).) This evidence, taken in conjunction with our evidence that burimamide stimulates acid secretion in the previously unstimulated stomach, might suggest a partial agonist action of burimamide. There are no reports in the literature of burimamide having a partial agonist action. However, it may be that burimamide is acting through release of some intermediary substance. In the anaesthetized cat, injection of burimamide causes a rise in arterial blood pressure; a fall in blood pressure would be expected if it were acting as a general histamine releaser. Histamine itself, if injected in a single intravenous injection, does not usually result in acid secretion but would produce a profound reduction of blood pressure. Therefore, there is little evidence that the stimulating action of burimamide reported here is due to a general release of histamine. There is some evidence that burimamide increased the rat gastric mucosal histamine output (Main & Whittle, 1974) and it is possible that this could influence gastric acid secretion.

Albinus & Sewing (1973) have shown that burimamide increased arterial blood pressure and heart rate, and decreases histamine-stimulated acid secretion in the anaesthetized cat. The increase in heart rate was abolished by propranolol, and the inhibition of histamine-stimulated acid secretion partly reversed by phenoxybenzamine. This evidence is consistent with the action of burimamide being explained, at least in part, by release of catecholamines. However, this would not explain the acid secretory stimulation, since catecholamines reduce gastric acid secretion (Jacobson, Linford & Grossman, 1966; Albinus & Sewing, 1973). There is no available evidence as to whether burimamide releases any other acid secreting agonist such as gastrin or acetylcholine.

Metiamide, in doses which produce significant reduction of pentagastrin-stimulated gastric acid secretion, does not produce an increase in acid secretion when injected under basal conditions (Figures 1 and 2). It, therefore, does not share the stimulant action of burimamide in the cat. Metiamide has been reported to be five to ten times more effective than burimamide as an H_2-receptor antagonist (Black *et al.*, 1973). This difference might, in part, be explained by a partial agonist action of burimamide. However, the doses of metiamide and burimamide used in this study were not of equal inhibitory potency (Black *et al.*, 1973) and experiments, as yet, have not been carried out using ranges of doses. Without this information it is difficult to discuss partial agonism.

We have previously reported (Reed, Smy, Venables & Harris, 1973) that burimamide injected in the unstimulated anaesthetized cat does not produce gastric acid secretion. However, these results were obtained in the later parts of experiments in which secretion had previously been stimulated by histamine, gastrin or pentagastrin infusion. Therefore, it seems likely in view of our current evidence that previous secretion, and possibly the associated phenomenon of tachyphylaxis to pentagastrin, had disguised this effect.

It is not surprising that there is a rise in MBF associated with the rise in acid secretion following burimamide injection (Figure 1). Ample evidence shows that the rise in acid secretion produced by a wide variety of stimulants is accompanied by an increase in MBF (Jacobson *et al.*, 1966; Harper *et al.*, 1968, Reed & Sanders, 1971). However, the mean Δ MBF/ΔH^+ i.e.

$$\frac{\text{the increased MBF above basal rate (ml/15 min)}}{\text{the increased } H^+ \text{ secretion above basal rate } (\mu Eq/15 \text{ min})}$$

during the peak burimamide-stimulated acid secretion was 0.065 ± 0.02. This represents the lowest mean ratio we have observed to any stimulant, the previous lowest recorded ratio being 0.087 seen during pentagastrin stimulation. Furthermore, we have reported that in the cat the Δ MBF/Δ H^+ seen during histamine stimulation with concomitant H_1-site blockade with mepyramine is considerably greater than that observed during pentagastrin stimulation. Jacobson & Chang (1969) have suggested that the excessive blood flow relative to acid secretion, which they have also reported to occur during histamine stimulation, is due to a direct effect of histamine on the gastric vasculature in addition to an acid secretory-linked hyperaemia. It might, therefore, be argued, since burimamide produces less vasodilatation per unit acid secretion than does histamine, that this suggested histamine-sensitive receptors on both the parietal cell and the gastric vasculature, the former being stimulated preferentially by burimamide.

However, we have reported (Reed & Smy, 1971) that the Δ MBF/Δ H^+ ratio is proportional to the dose of histamine infused i.e. the Δ MBF/Δ H^+ during infusion of histamine acid phosphate 4 5 $\mu g\ kg^{-1} min^{-1}$ is lower than that during infusion of 45 $\mu g\ kg^{-1} min^{-1}$. Since acid secretion is also related to the dose of histamine and since the acid secretory response to burimamide is lower than that seen to the lower dose of histamine, any suggestion of separate parietal and gastric vascular H_2-receptors must be tentative.

The acid stimulating action of burimamide in the resting stomach reported here in the cat, was not seen in the Heidenhain pouch dog (Black *et al.*, 1972) at doses of up to 10 mg/kg intravenously. This suggests either a species difference between cat and dog or possibly a difference between the responsiveness of the denervated pouch and the intact stomach Comparison of Figures 1 and 2 shows little difference between the acid responses in the anaesthetized and conscious animals. Moreover, the responses in anaesthetized cats were obtained in acutely splanchnectomized and vagotomized animals, whereas the conscious animals had intact gastric innervation. It, therefore, seems that it is more likely to be a species difference although to which species and which H_2-receptor sites it is limited, remains to be seen.

Finally, it has not previously been established whether part of the inhibitory effect of burimamide and metiamide is due to an action on the gastric mucosal barrier, i.e whether these drugs allow a greater back diffusion of H^+ and therefore only apparently cause inhibition of acid secretion. It is clear from the results reported here (Figure 3) that metiamide causes no such effects over the pH range which is seen during gastric acid secretion. The effect of burimamide on back diffusion could not be established because this drug stimulates acid secretion

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THE FAILURE OF *p*-CHLOROPHENYLALANINE TO AFFECT VOLUNTARY ALCOHOL CONSUMPTION IN RATS

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1 The effects of *p*-chlorophenylalanine (PCPA) administered orally and intraperitoneally on rat brain 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) content were compared. The depletion of brain 5-HT and 5-HIAA following PCPA (316 mg/kg) injected intraperitoneally every third day was not significantly different from that following the administration of PCPA (316 mg/kg) by stomach tube on eight consecutive days.

2 Rats tested for alcohol preference before, during and after treatment with PCPA on two intraperitoneal dose regimens (either 316 mg/kg then 100 mg/kg four days later or 316 mg/kg three times at intervals of three days) showed no reduction in voluntary alcohol consumption.

3 The results indicated that depletion of brain 5-HT and 5-HIAA is not responsible for the reduction of voluntary alcohol intake which has been reported to follow chronic oral administration of PCPA to the rat (Myers & Veale, 1968). The possibility of a learned aversion to alcohol due to an association with PCPA administration is discussed.

Introduction

Several reports have suggested a relation between cerebral 5-hydroxytryptamine (5-HT) and voluntary alcohol consumption in rats. Ahtee & Eriksson (1972) found that rats, genetically selected for their alcohol preference, had higher brain concentrations of 5-HT and its major metabolite, 5-hydroxyindoleacetic acid (5-HIAA) than rats that preferred to drink water. When rats were treated with *p*-chlorophenylalanine (PCPA) to lower cerebral 5-HT (Koe & Weissman, 1966) and tested for consumption of tap water and alcohol their alcohol intake was reduced after drug treatment (Myers & Veale, 1968; Cicero & Hill, 1970; Nachman, Lester & Le Magnen, 1970; Veale & Myers, 1970). Myers & Veale (1968) found that this effect occurred not only during the drug administration but was maintained up to two months after PCPA treatment ceased, when presumably the brain 5-HT concentration had returned to normal. PCPA also decreased alcohol consumption by the rat under conditions in which intake normally increased (Veale & Myers, 1970; Myers, Evans & Yaksh, 1972; Myers, 1972). As well as PCPA, *p*-chloroamphetamine depleted brain 5-HT concentrations and reduced the frequency of selection of 3%, 5% or 10% alcohol solutions when offered as a choice with tap water (Frey, Magnussen & Nielsen, 1970). This decreased intake also lasted after the drug treatment had ended.

In these tests for consumption of increasing concentrations of alcohol, PCPA has been administered daily for ten days or more by stomach tube in doses of 200 mg/kg to 300 mg/kg. Only Frey *et al.* (1970) measured brain 5-HT content at the end of oral administration of PCPA (300 mg/kg) during alcohol preference tests and found the concentration was reduced to 20% of the normal value. To determine if changes in cerebral 5-HT metabolism are crucial in regulation of voluntary alcohol consumption, the time course and magnitude of 5-HT depletion following PCPA administration must be determined over the extended time periods required for alcohol preference testing. Koe & Weissman (1966) reported that four or five daily oral doses of PCPA (100 mg/kg) or a single intraperitoneal dose of PCPA (316 mg/kg) resulted in the depletion of brain 5-HT to 7-12% of the control value after three to four days. Shillito (1970) has found that reduced concentrations of brain 5-HT can be maintained with intraperitoneal injections of PCPA at three day intervals. The following experiments were carried out to examine (1) the effects of oral and intraperitoneal administration of PCPA on brain 5-HT and 5-HIAA content with and without alcohol preference testing; and (2) the effect of intraperitoneal administration of PCPA on voluntary alcohol consumption.

Methods

The animals used were male albino rats of the Wistar strain supplied by A. Tuck & Son (Laboratory Animal Breeding Station, Rayleigh, Essex); they weighed about 150 g at the start of each experiment. The rats were housed in individual cages with food and water *ad lib.* They were kept under reversed daylight conditions in which a red light was on from 10 h 00 min to 22 h 00 min and white light for the remaining 12 hours The animals were adapted to the reversed daylight conditions for at least a week before any experiments were begun. All routine maintenance, fluid intake and weight readings, PCPA injections and decapitations for brain 5-HT and 5-HIAA estimations were carried out between 09 h 30 min and 10 h 00 minutes.

Drug administration

PCPA was administered orally by gastric tube in daily doses of 316 mg/kg. The PCPA (31.6 mg/ml) was suspended by agitation in 20 ml of 0.9% w/v NaCl solution (saline) acidified to pH 1.0 with 0.1M HCl and then the pH was raised to 4.5 with 0.1M NaOH as described by Myers & Veale (1968). When intraperitoneal injections of PCPA (316 mg/kg or 100 mg/kg) were given, the drug (31.6 or 10 mg/ml) was suspended in 1.0% or 0.5% Tween 80.

5-Hydroxytryptamine and 5-hydroxyindoleacetic acid estimation

5-HT and 5-HIAA in the brains of rats were determined fluorimetrically (Ahtee, Sharman & Vogt, 1970) at various intervals after treatment with PCPA. Control values were obtained from untreated animals. All rats were anaesthetized with chloroform and decapitated. The brain was quickly removed from the skull and the pineal body and olfactory lobes were dissected away. 5-HT and 5-HIAA were estimated in the precollicular forebrain.

Alcohol preference test

Rats were tested for alcohol preference using a two-choice, three position method as described by Myers & Holman (1966). The rats were given three graduated drinking tubes, one of which contained water, one alcohol and one was left empty. The position of the tubes was changed at random to counteract any position preference which the rats might develop for the tubes regardless of which fluid they contained. Ethanol solutions were prepared by dilution of 99.9% ethanol with tap water. The ethanol concentrations (v/v) were increased over eight consecutive days from 3 to 25% (3, 5, 7, 9, 11, 15, 20, 25%). Animals were offered only water for three days between each complete eight day testing sequence. No stress was used to induce the rats to drink alcohol, the test was only on voluntary alcohol consumption.

Brain 5-hydroxytryptamine and 5-hydroxyindoleacetic acid when no alcohol was offered

The effects of PCPA given orally or intraperitoneally during eight days were compared in 36 rats divided into three groups. One group ($n = 12$) was given up to eight daily doses of PCPA (316 mg/kg) by stomach tube and four animals were killed for 5-HT assays on days 3, 5 and 9 of the experiment. In the second ($n = 16$) and third ($n = 8$) groups PCPA was administered intraperitoneally; group 2 received the lower dose regimen of 316 mg/kg on the first day and an additional 100 mg/kg four days later; group 3 received the higher dose regimen of 316 mg/kg on the first, fourth and seventh days. 5-HT was estimated for group 2 on days 3, 5, 7 and 9 and for group 3 on days 5 and 10.

Brain 5-hydroxytryptamine and 5-hydroxyindoleacetic acid during alcohol preference tests

Cerebral 5-HT and 5-HIAA were assayed in rats ($n = 12$) tested for alcohol preference before and during the lower dose regimen of intraperitoneal injections of PCPA. All animals in this group were given a baseline eight day preference test at the end of which four animals were killed for 5-HT and 5-HIAA estimations. PCPA was administered to the remaining animals on day 2 of the three 'water-only' days and four days later during the second test sequence. 5-HT and 5-HIAA were determined on the fourth and eighth day after the first injection.

The effect of intraperitoneal injections of p-chlorophenylalanine on voluntary alcohol consumption

This was determined in four groups of eight animals. Following the baseline eight-day preference test two groups were injected with PCPA using the lower dose regimen. The first group of rats were injected with PCPA in 1.0% Tween 80; all following tests were carried out with PCPA suspended in a 0.5% Tween 80 solution. A third group received PCPA on the higher dose regimen In all three groups drug treatment began on the second 'water-only' day. The fourth group, a vehicle control group, received 0.5% Tween 80 in the same volume and at the same time intervals as the animals treated on the lower dose regimen Brain 5-HT and 5-HIAA concentrations were estimated at the end of the three alcohol preference test sequences.

Results

Brain 5-hydroxytryptamine and 5-hydroxyindoleacetic acid concentrations following p-chlorophenylalanine administration

The results of oral and intraperitoneal administration of PCPA on brain 5-HT and 5-HIAA are shown in Table 1. Three days after a single intraperitoneal injection or two successive oral doses of PCPA (316 mg/kg), the cerebral 5-HT content was significantly reduced to 8.4% ($P < 0.01$) and 10.6% ($P < 0.01$) of the control value respectively. This 5-HT depletion was not different in the two modes of administration. Two additional days of oral administration reduced the 5-HT values to 3.6% of control. At this time animals on the lower intraperitoneal PCPA dose regimen had had no further drug treatment and the cerebral 5-HT content was 10.3% of control. However, with the higher dose regimen, the 5-HT concentration was 5.7% of the control, a value not different from that of the orally treated group, but significantly less than that after the lower dose regimen ($P < 0.05$). Twenty-four hours after the eighth and final oral dose of PCPA, the cerebral 5-HT content was found to be 3.0% of the control. This was significantly less than after the lower intraperitoneal dose regimen (17% of control; $P < 0\ 001$), but not different from the values after the high dose intraperitoneal regimen (4 7% of control). In all cases in which 5-HIAA was measured after PCPA treatment, the acid metabolite was reduced and was often not detectable.

Table 2 shows the results of the lower intraperitoneal dose regimen of PCPA and of Tween 80 control injections on the 5-HT and 5-HIAA concentrations during alcohol preference testing. Immediately following the first alcohol preference test, the 5-HT and 5-HIAA values did not differ from those of controls. PCPA treatment significantly reduced the brain concentrations of 5-HT and 5-HIAA during the second alcohol preference test. At the conclusion of the third preference test the concentration of 5-HT was within the normal range, while that of 5-HIAA was significantly greater than the control value ($P < 0.01$) At the end of the three alcohol preference test sequences animals injected only with 0.5% Tween 80 had cerebral 5-HT and 5-HIAA concentrations similar to those of untreated animals.

Table 1 Effects of intraperitoneal and oral *p*-chlorophenylalanine (PCPA) administration on rat brain 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) content in rats drinking only water

Days of experiment	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
i.p. PCPA (mg/kg)	316				100					
5-HT			37 ± 6* (8.4)		45 ± 1* (10.3)		36 ± 1* (8.2)		74 ± 6* (17.0)	
i.p. PCPA (mg/kg)	316			316			316			
5-HT					25 ± 8* (5.7) *n* = 8					20 ± 3* (4.7) *n* = 8
5-HIAA					<10*					<10*
oral PCPA (mg/kg)	316	316	316	316	316	316	316	316		
5-HT			45 ± 3* (10.6)		16 ± 2* (3.6)				13 ± 4* (3.0)	
5-HIAA			69 ± 2* (36.5)		<10*				<10*	

Each 5-HT and 5-HIAA value is the mean with s.e. mean of four estimations (except where specified) expressed as ng/g brain wet weight and corrected for recovery. Control values. 5-HT, 436 ± 25 ng/g (17 estimations); 5-HIAA, 190 ± 14 ng/g (18 estimations).

* Significantly ($P < 0.01$) different from control value.

Numbers in parentheses are the mean estimates expressed as percentages of the control values.

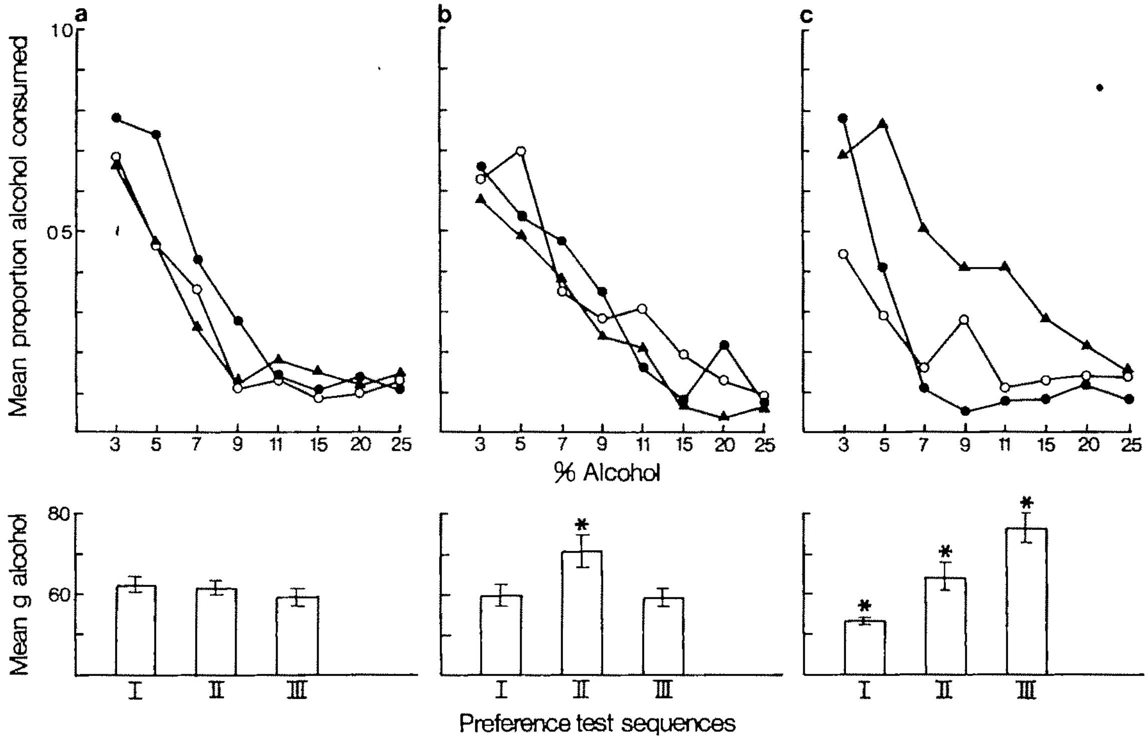

Figure 1 Upper panels: mean proportion of alcohol solution drunk to total daily fluid intake for increasing concentrations of alcohol offered during three preference test sequences: (●) 1st sequence; (○) 2nd sequence; (▲) 3rd sequence. Each point represents the mean for eight animals.

Lower panels. mean amount of alcohol consumed (in g) during each preference test sequence. Each histogram represents a mean of 64 observations Vertical lines show s.e. mean. (a) 0.5% Tween 80, injected twice and in proportionate volume as in b (vehicle control). (b) *p*-Chlorophenylalanine (PCPA) in 0.5% Tween 80, 316 mg/kg and 100 mg/kg four days later. (c) PCPA in 0.5% Tween 80, 316 mg/kg x3 at three day intervals. In each case the initial injection was given one day before alcohol preference test sequence II began, when only water was offered

Upper panels only the 3rd sequence (▲) values in (c) are significantly different ($P < 0.01$) from other test sequences within the series.

Lower panels: * indicates significant difference ($P < 0.01$) from other test sequences within each series of three.

Table 2 Effects of intraperitoneal administration of *p*-chlorophenylalanine (PCPA) and 0.5% Tween 80 on rat brain 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) during alcohol preference testing

		End of Test I	*Test II Day 3*	*Test II Day 7*	*End of Test III*
PCPA†	5-HT	463 ± 17	29 ± 4	49 ± 6	510 ± 23
	5-HIAA	192 ± 7	<10	16 ± 1	297 ± 22
0.5% Tween 80†	5-HT				393 ± 15
	5-HIAA				164 ± 8

Each 5-HT and 5-HIAA value is the mean with s e. mean of four estimations, expressed as ng/g brain wet weight and corrected for recovery.

†PCPA, 316 mg/kg, was administered i.p. on the second day of the three day interval between the alcohol preference test sequences I and II. In this interval the rats were given only water to drink A second dose of PCPA, 100 mg/kg, was administered four days later, i.e. on the third day of the second alcohol preference test sequence.

0.5% Tween 80 (i.p) was administered on the same time course in a volume of 10 ml/kg per injection.

Alcohol consumption

Figure 1 shows the mean proportion of total daily fluid consumed as an alcohol solution (upper panel), and mean alcohol intake expressed in grams (lower panel) during three test sequences. Control animals treated with 0.5% Tween 80 only (Figure 1a) showed no change in either the proportion of alcohol or grams of alcohol consumed before, during or after the intraperitoneal injections. Figure 1b shows the effect on alcohol consumption of the lower intraperitoneal dose regimen of PCPA in 0.5% Tween 80. In the second test sequence, during the period of drug treatment, the mean proportion of the daily fluid intake taken in the form of alcohol solution was not significantly different from that of the pre- and post-drug sequences. However, during the second test sequence, the average daily fluid intake increased so that the mean total amount of alcohol consumed by the rats did increase to a significant ($P < 0.05$) extent. A rise in the weight of alcohol consumed, but not in the proportion of alcohol solutions consumed was also obtained in a group of eight rats in which 1% (instead of 0.5%) Tween 80 was used to suspend the PCPA.

With the higher dose regimen of PCPA (Figure 1c), the mean proportion of fluid consumed as alcohol was the same in the tests before and during PCPA treatment, but increased significantly ($P < 0.01$) during the post-drug preference test. The mean intake in grams of alcohol increased significantly with each successive sequence ($P < 0.01$).

The results were tested statistically using an analysis of variance.

Discussion

The present results show that two or three intraperitoneal injections of PCPA separated by up to four days can cause as large a depletion of cerebral 5-HT and 5-HIAA as eight successive daily doses given orally. Intraperitoneal administration reduced the number of treatments given to each animal and was not accompanied by any reduction of food and water intake or by weight loss. In fact, the total intake of fluid was increased following the intraperitoneal injection of PCPA. This may reflect an attempt to dilute the PCPA in the peritoneal cavity. However, the volume of alcohol solution consumed expressed as a proportion of the total daily fluid intake remained similar to that observed during the pre-drug testing sequence despite the increased total fluid intake. Only in the period following the high dose regimen of PCPA was there a significant increase in the proportion of alcohol solution consumed.

Although there was a similar depletion of cerebral 5-HT following both oral and intraperitoneal administration of PCPA, no decrease in the selection of alcohol was observed. This suggests that depletion of cerebral 5-HT is not responsible for the changes in alcohol selection reported by Myers & Veale (1968). The difference between the present findings and the results presented by Myers & Veale (1968) appear to be related to the route and time of administration of PCPA and might be explained by the 'conditioned aversion' that occurs when an unpleasant stimulus becomes associated with a rewarding condition, so that the reward is then avoided. In this case, the irritant effects of orally administered PCPA produced avoidance of the alcohol which the rats had chosen to drink before treatment. That repeated oral administration of PCPA has deleterious effects is demonstrated by the fall in body weight reported by Myers & Veale (1968), an observation which was confirmed by our own experiments. Such learned associations have been reported by Nachman (1970) and by Green & Garcia (1971) to occur when milk, grape juice or saccharin was presented to rats prior to treatment with lithium chloride or apomorphine. Nachman *et al.* (1970) found that when rats were injected with PCPA within 5 min of a baseline test for intake of 6% alcohol solution there was a decrease in their subsequent consumption of alcohol.

Similar results were obtained when *n*butyraldoxime, pyrazole or lithium chloride was injected in place of PCPA and when the drugs were tested against the consumption of saccharin solution. These authors suggested that the rats avoided drinking because of an association of the irritant effects of the drugs with drinking. Green & Garcia (1971) have emphasized the importance of the temporal association between the injection of the drug and the presentation of the test solution in the development of such a learned aversion. A solution which was offered before and just after an injection of apomorphine was later rejected by rats, whereas the intake of a fluid which was offered during recuperation from the effects of the injection showed an increase over baseline values. A delay of 30 min between the injection and the presentation of the test fluid could still cause this effect.

In the present experiments there was a 48 h delay between the initial injection of PCPA and the start of the alcohol preference test. This may have prevented the rats from associating the presentation of alcohol with treatment with PCPA.

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PROSTAGLANDIN PRODUCTION BY A MILD INFLAMMATORY LESION IN SHEEP

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By implanting a Teflon chamber subcutaneously into a sheep a mild aseptic inflammatory lesion was produced from which wound fluid could be sampled daily. The prostaglandin content of this wound fluid was examined. Prostaglandin E_2 activity was found in all samples, and a peak was apparent on the fourth or fifth day after producing the lesion The amount of prostaglandin activity appeared to be unrelated to the total number of polymorphs in the fluid or to the volume of this exudate.

Introduction Prostaglandins have been detected in fluid from several types of lesion, but in few species. The presence of prostaglandin E_2 was demonstrated in the exudate from a carrageenin-air bleb, in rat subcutaneous tissue (Willis, 1970) and in lymph from scalded dogs' paws (Anggard & Jonsson, 1971). Prostaglandin E_1 and smaller amounts of E_2 were found in the aqueous humour of rabbit eyes in which uveitis had been induced (Eakins, Whitelocke, Perkins, Bennett & Unger, 1972), and prostaglandins E and F were recovered from inflamed human skin (Greaves, Sondergaard & McDonald-Gibson, 1971). Rat pleural and peritoneal exudates induced by carrageenin have contained prostaglandins E and F, as has synovial fluid in rheumatoid arthritis patients undergoing anti-inflammatory therapy (Velo, Dunn, Giroud, Timsit & Willoughby, 1973).

There must inevitably be some discussion about species differences in the prostaglandin types produced, and, within those species, about the cells which produce them. In the experiments described here, relatively large volumes of sterile wound fluid have been obtained from the subcutaneous tissues of the sheep. As well as prostaglandins, the cell content of this fluid has been studied.

Methods Wound fluid was collected from sheep with a perforated Teflon or stainless steel cylinder (Greenwood, 1970) of 29 mm external length and 19 mm external diameter. One end of the chamber was closed, while a catheter passed through a lid at the other end so that its tip lay centrally in the chamber. The cylinder was placed subcutaneously in the shoulder region beneath the *M. cutaneus colli.* The muscle layer and skin were closed over the chamber, the catheter passed out through the top of the incision and was held in place by Michel clips and sutures. The catheter remained plugged except when wound fluid samples were being taken.

The wound fluid was removed every 24 hours. The catheter was kept clear between sampling by injecting a small quantity of heparinized 0.9% w/v NaCl solution (saline). Each collection continued until no further fluid was obtained. Blood samples were taken from the jugular vein at the same time. Total and differential white blood cell counts were carried out on both wound fluid and blood. Evidence of bacterial contamination was sought, but not found, by culturing each wound fluid sample on blood agar.

The prostaglandins were extracted by acidifying the wound fluid to pH 3 with HCl before extracting the sample twice with equal volumes of ethyl acetate. The ethyl acetate was evaporated to dryness and the residue dissolved in 1.0 ml of Krebs bicarbonate solution. The prostaglandin content of each extract was assayed in terms of prostaglandin E_1 equivalents on the rat fundic strip preparation (Vane, 1957). The tissue was superfused at 5-10 ml/min with Krebs bicarbonate solution, containing mepyramine maleate (0.1 mg/l), hyoscine hydrobromide (0.1 mg/l), propranolol (3 mg/l), phenoxybenzamine (0.1 mg/l), methysergide (0.2 mg/l) and indomethacin (1 mg/l). Evidence for the identification of individual prostaglandins in the extracts was obtained by thin layer chromatography, with the AI and AII systems of Gréen & Samuelsson (1964).

Results Daily determinations in two individual sheep (Figure 1) showed that the wound fluid volumes and total leucocyte counts progressively rose and then declined, whereas total prostaglandins remained comparatively steady save for one outstanding peak. In sheep (a) the peak occurred on day 5, and in sheep (b), on day 4. In neither animal did the peak coincide with the peak total polymorph count. In one sheep the prostaglandins peaked one day later than the total polymorphs, while in the other they peaked two days earlier. The peak polymorph count in both

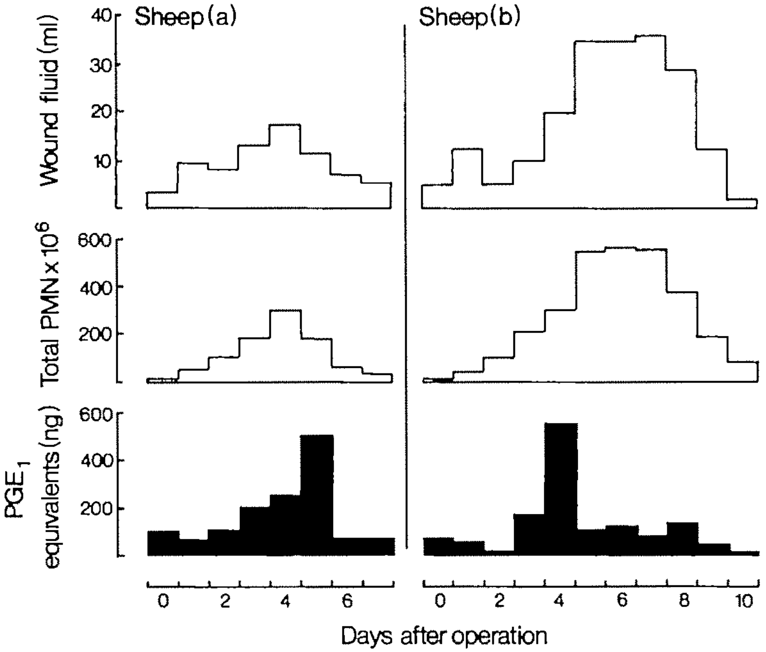

Figure 1 Volume, total polymorphonuclear leucocyte (PMN) count, and total prostaglandin E$_2$-like activity (expressed in ng quantities in terms of prostaglandin (PG) E$_1$ equivalents) of wound fluid from a mild aseptic surgical lesion in each of two sheep.

animals was associated with a low total prostaglandin level.

Discussion Previous (unpublished) experiments have shown that mixed populations of sheep blood leucocytes can produce prostaglandins when incubated and that this production can be increased tenfold by adding dead staphylococci to the cultures. Granulocytes from the rabbit peritoneal cavity behave similarly with respect to *B. pertussis* (Higgs & Youlten, 1972). It may well be that the dominant variable in producing the prostaglandin peak is not the total number of polymorphs in the wound, but the change in stimulus to prostaglandin synthesis by these cells.

The cell population in the wound fluid may not accurately represent the polymorph accumulation in the surrounding tissues. Biopsies do not, however, indicate that high numbers of polymorphs are present in the tissues at this time. Small numbers of monocytes, which, unlike polymorphs, are more obvious in the surrounding tissues than in the wound fluid, are beginning to accumulate. Were the prostaglandin peak to be related to the accumulation of monocytes, it might be expected that the rise would be prolonged and progressive.

An alternative explanation of the peak might be that prostaglandin removal or destruction is briefly diminished for some reason. It is, perhaps, surprising that prostaglandins remain in such large quantities in the wound fluid, and are not almost wholly removed by the blood, for equilibration of small molecules between wound fluid and blood appears to be good. On the other hand some degree of stasis is suggested by the P_{CO_2} and P_{O_2} levels of the wound fluid, which have been measured in other experiments. The restoration of prostaglandins following daily removal of wound fluid from the tissue space, indicates continuous active production.

Thin layer chromatography showed that the prostaglandin-like activity moved almost entirely with the prostaglandin E$_2$ standard (smooth muscle stimulating material which travelled with prostaglandin E$_1$ or F$_{2\alpha}$ was detected in trace amounts in only a few of the samples assayed). Incubated sheep mixed leucocytes produce predominantly E series prostaglandins. Velo *et al.* (1973) suggested that prostaglandin E$_2$ increases vascular permeability, but there was no correlation between the total prostaglandin content or concentration, and the volume of wound fluid. Of course, increased permeability may facilitate the removal of wound fluid if the tissue pressure builds up adequately. In these experiments tissue fluid pressure is certainly positive, but the lax tissue in which the chamber is implanted, and the large volumes of wound fluid recovered do not suggest that great quantities of wound fluid are being forced back into the blood vessels.

The sources of prostaglandin E_2 in this lesion are being studied That it is unlikely to arise from the tissue acutely injured at operation is suggested by the delay in maximum prostaglandin production. Our findings that sheep mixed leucocytes produce prostaglandin E indicate that the polymorph population is likely to contribute. This production is minimal unless bacteria are present, and these were not found in the wound fluid. However, there is bound to be some material suitable for phagocytosis in the damaged tissue, if, indeed, phagocytosis stimulates prostaglandin production by leucocytes as Higgs & Youlten (1972) have suggested.

We are grateful to Dr L.J.F. Youlten and G A. Higgs, Esq., for drawing our attention to the presence of prostaglandins in previous sheep wound fluid samples. P J.K. is a Research Training Scholar of the Wellcome Trust.

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KINETICS OF AGONIST CONDUCTANCE CHANGES DURING HYPERPOLARIZATION AT FROG ENDPLATES

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The effect of rapid hyperpolarization on acetylcholine- or carbachol-induced currents was studied at voltage clamped frog endplates Following a hyperpolarizing step the agonist-induced conductance increased approximately exponentially to a new level. The rate constant for this process was smaller during hyperpolarization or lowered temperature, and was also smaller for acetylcholine than for carbachol. The results are interpreted in terms of a potential-dependent conformational change of the receptor

Introduction The conductance change produced by agonists acting at the endplate membrane can be defined as $\Delta I/(V - E)$, where ΔI is the additional current produced by the agonist, V is the membrane potential and E is the equilibrium potential for the agonist action. Over a range of potentials from +50 mV to −180 mV the equilibrium conductance change produced by iontophoretic or bath application of various agonists increases exponentially with membrane hyperpolarization, an *e*-fold change being obtained with hyperpolarizations of the order of 100 mV (unpublished observations) Furthermore, the time constant for decay of endplate currents also increases exponentially with hyperpolarization (Magleby & Stevens, 1972). A simple model which accounts for these observations is to suppose that in the presence of an agonist the receptors distribute between inactive (R) and active (R*) forms, which interconvert with voltage-dependent rate constants f and b.

$$R \underset{b}{\overset{f}{\rightleftharpoons}} R^*$$

Under these circumstances following a step change in membrane potential the new agonist conductance should develop exponentially with a rate constant (f + b).

Methods The experiments were performed on isolated sartorius muscles of *Rana temporaria*. Acetylcholine or carbachol was applied iontophoretically to sensitive endplate regions from single or twin pipettes. A conventional two electrode voltage clamp technique was used. However, to obtain sufficient temporal resolution several improvements were necessary. Firstly, in order to change the membrane potential as rapidly as possible the output amplifier of the feedback system was an Analog Devices 171J giving an output swing of ±140 V. Aluminium foil wrapped around the intracellular voltage recording electrode was connected to the output of its unity gain voltage follower. Although the total clamp current was monitored in the usual way, this was not considered to give a perfectly reliable measure of the extra agonist current at early times following steps, partly because the area reached by agonist may not be perfectly isopotential, but mainly because this record is contaminated by current flowing to charge distant parts of the fibre. If the length constant of the endplate changes during agonist action it may not then be valid to subtract this current to obtain the agonist-induced current. Therefore the membrane current in the region acted upon by the agonist was taken to be proportional to the voltage drop in the Ringer solution just outside this region. This voltage drop was measured with an extracellular NaCl-filled microelectrode placed just outside the membrane as close to the tip of the iontophoretic pipette as was practical. This electrode was also 'cathodally screened' as above. Finally, most of the experiments were conducted at low temperatures (2°-6°C) achieved by the use of two Peltier elements (Mectron Frigistor) controlled by feedback from a bath thermistor The bath temperature was separately monitored with an electronic thermometer (Cormark 1601). The records were photographed with simultaneous fast and slow time bases. All four microelectrodes were placed with their tips within a length equal to 1-2 fibre diameters. The iontophoretic pipette tip was positioned close to the endplate membrane so as to limit the area activated. The Ringer solution contained (mM): Na, 112; Cl 118.1; K 2.5; Ca 1.8; Tris 5; the pH was 7.4.

Results With the present system, step hyperpolarizations of 100 mV could be achieved in 50 μs (checked by inserting the extracellular microelectrode). However, the extracellular field (taken to be proportional to membrane current) did not 'settle' until 100-200 μs following the

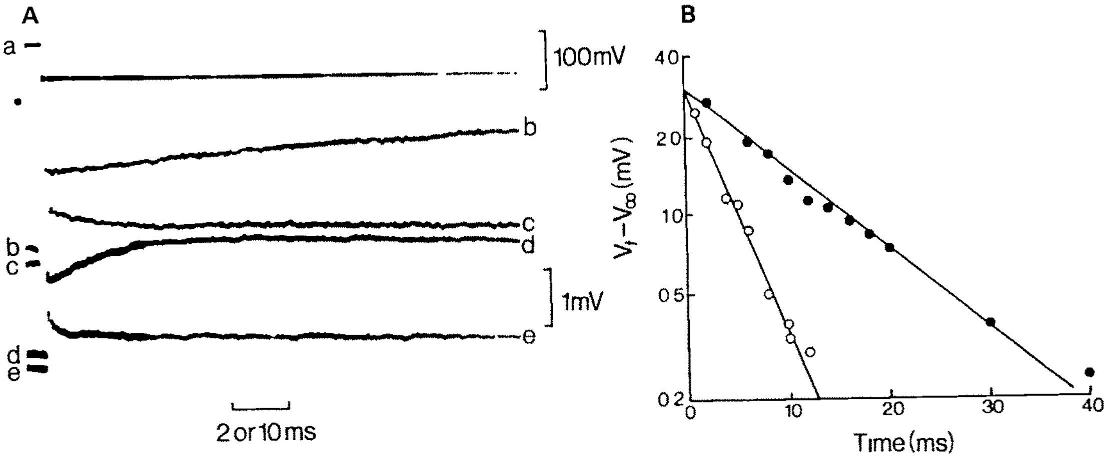

Figure 1 (A) Effect of sudden hyperpolarization on extracellular field potentials produced by iontophoretic acetylcholine application. (a) Shows the membrane potential; (e) and (d) show the extracellular field potential produced by the step hyperpolarization in the absence (e) and presence (d) of acetylcholine; (c) and (b) are the same as in (e) and (d) photographed simultaneously with a faster time base (B) The onset of the increase in agonist-induced conductance produced by hyperpolarization. V_t is the difference in the extracellular field potential established by a step hyperpolarization from −80 to −155 mV in the presence and absence of agonist at various times following the step. V_∞ is the steady difference in field potential reached at $t = 100$ ms. $(V_t - V_\infty)$ is plotted semilogarithmically on the ordinate scale, and the abscissa scale is time elapsed from the step. V_t is assumed proportional to I_t (see text). Acetylcholine (●) or carbachol (○) were applied iontophoretically to the same endplate spot from separate barrels of a twin pipette The solid lines have slopes of 74 s^{-1} (acetylcholine) and 216 s^{-1} (carbachol).

command, which therefore constituted a 'dead time' during which no measurement was available. In the absence of agonist the 'current' record then remained almost steady for the duration of the step (100 ms), though there was often an early dip. During the action of either acetylcholine or carbachol the membrane current was at all times larger, and consisted of a step that was apparently instantaneous followed by a further slow increase (Figure 1A). The agonist-induced current was obtained by measurement and subtraction of currents obtained in the presence and absence of agonist. The slow increase in current was apparently exponential, since if log $(V_\infty - V_t)$, where V_t is the extra field potential at time t and V_∞ the final steady potential, was plotted against t a straight line was obtained (Figure 1B). The slope of this line gave an apparent rate constant k, which decreased with increasing hyperpolarization. For example in one experiment with acetylcholine the values of k following steps to 88, 130, 157 and 213 mV were 96, 70, 41 and 21 s^{-1} respectively, at 4° C. After correction to −100 mV assuming a voltage dependence of e-fold per 70 mV (the overall value seen in three experiments), the mean value of k obtained in 15 experiments with acetylcholine (2°-6° C) was $160 \pm 21\ ^{-1}$ (± s.e. mean). At room temperature (20° C) k was much larger (700 s^{-1}).

Finally, in three experiments acetylcholine and carbachol were compared at the same endplate patch. The carbachol-induced conductance increase following hyperpolarization occurred faster than that obtained with acetylcholine. In these experiments the ratio $k_{\text{carb}}/k_{\text{ACh}}$ was 2.38 ± 0.15 (mean ± s.e. mean). In one experiment at 20° C the values for k were 3.1 ms^{-1} (carbachol) and 0.7 ms^{-1} (acetylcholine) at −100 mV, giving a ratio of 4.4.

Discussion For a single site receptor the rate constant for increase in conductance is given by

$$k = f\,\frac{a}{K + a} + b$$

where a is the agonist concentration and K the equilibrium dissociation constant. It is assumed that in the present experiments the receptors are largely saturated ($a \gg K$) so that $k \approx (f + b)$. $(f + b)$ for acetylcholine determined in the present experiments is very similar to the value of b determined from decay of the endplate current (Magleby & Stevens, 1972) or from analysis of endplate noise (Katz & Miledi, 1972; Anderson &

Stevens, 1973) This implies that b is considerably greater than f, so that even when all the receptors are saturated by acetylcholine only a small fraction are in the active form. This finding agrees with indirect evidence from analysis of endplate current decay following neostigmine treatment (Katz & Miledi, 1973)

If the receptor were composed of a number of subunits each of which independently underwent a conformational change and more than one subunit had to be in the active conformation for the channel to be open, then the conductance increase following a step hyperpolarization should follow a sigmoidal path (Hodgkin & Huxley, 1952). On the other hand if there were a concerted transition of all subunits the conductance increase should be simply exponential; however in this case the rate constant would depend on a power of the agonist concentration. Although in a number of the present experiments in which acetylcholine alone was tested the scatter in the measurements was much less than shown in Figure 1B, no consistent deviation from exponentiality (at least in the direction expected for independence) has yet been observed. However, any deviation produced by independence of subunits would be very small in the present experiments, in which the fraction of receptors activated before applying the hyperpolarization was already significant compared to the fraction finally activated. Experiments with steps from much more positive holding potentials should resolve this question

The observation that conductance increases much faster when carbachol is used rather than acetylcholine is interesting for two reasons. Firstly, it lessens the possibility that the responses studied were artefacts. Secondly, the observed ratio of rate constants for carbachol and acetylcholine is similar to the ratio of channel lifetimes obtained by comparison of noise spectra (Katz & Miledi, 1972), implying that both methods probe essentially the same molecular event.

The present technique should be capable of considerable extension. For example a close comparison of b and (f + b) at a single endplate might permit an accurate estimate of f. Also, if binding is assumed very rapid compared to conformation changes, various possible models for receptor activation lead to simple, and testable, predictions for the dependence of the conductance change kinetics on agonist concentration.

I wish to thank K. Didcock and R. Croxton for help in the construction of the low temperature bath

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AN AMBIGUITY IN RECEPTOR THEORY

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Two expressions for calculating receptor occupancy by antagonist in competitive drug antagonism are contrasted

The following problem may arise when calculating receptor occupancy by an antagonist from standard 'occupation' theory (Gaddum, 1937) of competitive drug antagonism

If A and B represent concentrations of agonist and antagonist, K_1 and K_2 their respective affinity constants, y and z their respective occupied receptor fractions and x the dose-ratio, the equation $z = (x - 1)/x$ (1) is often used to determine the receptor fraction occupied by the antagonist. Equation (1) was originally formulated in respect of rate theory (Paton, 1961), but when the same equation is applied to arguments based on occupation theory the following difficulty arises.

It is possible to derive equation (1) by combining $K_2B = x - 1$ (2) and $z = K_2B/(K_2B + 1)$ (3), but equation (1) contradicts $z = (1 - y)(x - 1)/x$ (4) which follows from $K_1 = y/(1 - y)A = y/(1 - y - z)Ax$. However, the above derivation of equation (1), insofar as it applies to occupation theory, is based on premises which are inconsistent since (2) refers to the situation where both agonist and antagonist are present and in equilibrium with receptors (Schild, 1949) whilst (3) refers to the situation in which only the antagonist is present and in equilibrium with receptors By contrast, the derivation of (4) assumes the presence at equilibrium of both agonist and antagonist.

It would seem thus that, in terms of standard occupation theory, estimates of antagonist receptor occupancy by equation (1) do not represent the actual occupancy in a given experiment which is given by equation (4). However, (4) is difficult to evaluate because of the term containing y. Equation (1) could, however, be regarded as indicating receptor occupancy by antagonist if no agonist was present. Of course the results obtained by (1) and (4) could be very close, since the two equations converge when y becomes small, as is the case when an agonist of high efficacy acts on a preparation with abundant spare receptors. In such cases equation (1) is a very useful approximation to the result obtained from standard occupation theory.

I would like to thank Dr D.H. Jenkinson and Professor J.W. Black for discussion.

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JANUARY 1975

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British Journal of Pharmacology

VOLUME 53 NUMBER 2 FEBRUARY 1975

page

Systematic Pharmacology

157 KELLY, J.G. & SHANKS, R.G. Metabolic and cardiovascular effects of isoprenaline and salbutamol in the dog

163 PAOLONI, H.J. & WILCKEN, D.E.L. The action of aminophylline on the acutely transplanted dog heart. effect of α- and β-adrenoceptor blockade

173 DALY, M.J., FLOOK, JUDITH J. & LEVY, G.P. The selectivity of β-adrenoceptor antagonists on cardiovascular and bronchodilator responses to isoprenaline in the anaesthetized dog

183 McCALDEN, T.A. The inhibitory action of oestradiol-17-β and progesterone on venous smooth muscle

193 MARLEY, E. & NISTICÒ, G. Tryptamines and some other substances affecting waking and sleep in fowls

Autopharmacology

207 HAYASHI, E & YAMADA, S. Pharmacological studies on surugatoxin, the toxic principle from Japanese ivory mollusc (*Babylonia japonica*)

217 MAIN, I.H.M. & WHITTLE, B.J.R. Investigation of the vasodilator and antisecretory role of prostaglandins in the rat gastric mucosa by use of non-steroidal anti-inflammatory drugs

225 DONOVAN, B.T. Indomethacin, ketoprofen and corpus luteum regression in the guinea-pig

229 NUSTAD, K., PIERCE, J.V. & VAAJE, KIRSTEN. Synthesis of kallikreins by rat kidney slices

Pharmacokinetics

235 BOND, P.A., BROOKS, B.A. & JUDD, A. The distribution of lithium, sodium and magnesium in rat brain and plasma after various periods of administration of lithium in the diet

241 PARVEZ, SIMONE, PARVEZ, S.H. & YOUDIM, M.B.H. Variation in activity of monoamine metabolizing enzymes in rat liver during pregnancy

247 GARCÍA, A.G. & SÁNCHEZ-GARCÍA, P. Influence of cocaine and sodium on bretylium uptake by reserpine-treated guinea-pig left atrium

257 DA PRADA, M., OBRIST, R. & PLETSCHER, A. Discrimination of monoamine uptake by membranes of adrenal chromaffin granules

Drug Mechanisms

267 KALSNER, S. Role of extraneuronal mechanisms in the termination of contractile responses to amines in vascular tissue

279 CHIOU, C.Y. & MALAGODI, MARJORIE H. Studies on the mechanism of action of a new Ca^{2+} antagonist, 8-(*N,N*-diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride in smooth and skeletal muscles

287 GREEN, A.R., SOURKES, T.L. & YOUNG, S.N. Liver and brain tryptophan metabolism following hydrocortisone administration to rats and gerbils

293 HARRIS, D.W., SMY, J.R., REED, J.D. & VENABLES, C.W. The effects of burimamide and metiamide on basal gastric function in the cat

Psychopharmacology

299 HOLMAN, R.B., HOYLAND, VALERIE & SHILLITO, ELIZABETH E. The failure of *p*-chlorophenylalanine to affect voluntary alcohol consumption in rats

305 SHORT COMMUNICATIONS

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INDEX

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VOLUME 53(3) MARCH 1975

British Journal of Pharmacology

page

Systematic Pharmacology

317 CHAWLA, NARESH, JOHRI, M.B.L., SAXENA, P.N. & SINGHAL, K.C. Cholinergic mechanism in central thermoregulation in pigeons (SP2, AP1)

Autopharmacology

323 JONES, MARGARET E.L. & SPRIGGS, T.L.B. Noradrenaline and motor transmission in the vas deferens of the mouse (AP1)
333 MINATOYA, H. & SPILKER, B.A. Lack of cardiac or bronchodilator tachyphylaxis to isoprenaline in the dog (AP1)
341 BURDEN, D.T. & PARKES, M.W. An investigation of the tachycardia produced by intracerebroventricular injections of isoprenaline in mice (AP1, SP1, DM2)
349 SLOTKIN, T.A. Effects of reserpine on the adrenal medulla of the spontaneously hypertensive rat (AP1)
357 RUBIN, R.P. & WARNER, W. Nicotine-induced stimulation of steroidogenesis in adrenocortical cells of the cat (AP3, DM1)

Drug Mechanisms

363 ROBINSON, D.A., TAYLOR, J.G. & YOUNG, J.M. The irreversible binding of acetylcholine mustard to muscarinic receptors in intestinal smooth muscle of the guinea-pig (DM2, DM1)
371 HUGHES, J., KOSTERLITZ, H.W. & LESLIE, FRANCES M. Effect of morphine on adrenergic transmission in the mouse vas deferens. Assessment of agonist and antagonist potencies of narcotic analgesics (DM2)
383 COX, B.M., GINSBURG, M., WILLIS, JULIA & DAVIES, J.K. The offset of morphine tolerance in rats and mice (DM2, SP2)
393 NESHEIM, BRITT-INGJERD. Action of β-adrenoceptor antagonists on the response to isoprenaline in the oestrogen dominated rabbit uterus (DM2, AP1)
403 NESHEIM, BRITT-INGJERD, OSNES, J.-B. & ØYE, I. Role of cyclic adenosine $3',5'$-monophosphate in the isoprenaline-induced relaxation of the oestrogen dominated rabbit uterus (DM2, DM1, AP1)

Miscellaneous

409 EVANS, D.P. & THOMSON, D.S. Inhibition of immediate hypersensitivity reactions in laboratory animals by a phenanthroline salt (ICI 74,917) (M, AP2)

SHORT COMMUNICATIONS

419 ANLEZARK, G.M. & MELDRUM, B.S. Effects of apomorphine, ergocornine and piribedil on audiogenic seizures in DBA/2 mice (SP2, DM2, AP1)

422 BOWERY, N.G., BROWN, D.A. & COLLINS, J.F. Tetramethylenedisulphotetramine: an inhibitor of γ-aminobutyric acid induced depolarization of the isolated superior cervical ganglion of the rat (DM1, AP1)

425 AUNIS, D., MANDEL, P., MIRAS-PORTUGAL, M.T., COQUILLAT, G., ROHMER, F. & WARTER, J.M. Changes of human plasma dopamine-β-hydroxylase activity after intravenous administration of theophylline (DM1)

428 BOTTING, J.H. Sensitivity of neonatal rabbit ileum to histamine (AP2)

431P PROCEEDINGS OF THE BRITISH PHARMACOLOGICAL SOCIETY, London, 8-10 January, 1975.

Systematic Pharmacology

SP1	Cardiovascular system
SP2	Central nervous system
SP3	Other physiological systems

Pharmacokinetics

PK1	Storage, distribution, fate
PK2	Synthesis, metabolism
PK3	Uptake, release

Autopharmacology

AP1	Adrenergic, cholinergic transmission
AP2	Histamine, kinins, prostaglandins, etc.
AP3	Hormones

Drug Mechanisms

DM1	Molecular, cellular, biochemical, biophysical
DM2	Receptors, antagonism, potentiation, tolerance

CP	**Clinical Pharmacology, Experimental Therapy**
CT	**Chemotherapy**
M	**Miscellaneous**
PM	**Pharmacometrics**
PP	**Psychopharmacology**
TX	**Toxicology**

PROCEEDINGS OF THE BRITISH PHARMACOLOGICAL SOCIETY

London, 8-10 January, 1975

431P ENERO, MARIA, A. & LANGER, S.Z. Pharmacological effects of histamine on the isolated cat nictitating membrane.

432P HOLTON, PAMELA, IMPICCIATORE, MARIANINA & SPENCER, JAN. Dose response relationship and comparison of the secretory potency of methyl histamine and histamine on the isolated guinea pig stomach.

433P CURWAIN, B.P. & MILLS, G.A. Effects of atropine on gastric secretion and mucosal blood flow in conscious dogs.

434P McISAAC, R.L. & WILSON, G.M. (introduced by Pamela Holton). The effect of atropine on pentagastrin-induced gastric acid secretion and mucosal blood flow in the conscious rat.

435P BRIMBLECOMBE, R.W., DUNCAN, W.A.M., DURANT, G.J., GANELLIN, C.R., PARSONS, M.E. & BLACK, J.W. The pharmacology of cimetidine, a new histamine H_2-receptor antagonist.

436P FOSTER, R.W. & RAMAGE, A.G. Observations on the effect of dibenzoxazepine (CR) and N-nonoyl-vanillylamide (VAN) on sensory nerves.

437P CONSTANTI, A. & NISTRI, A. (introduced by Quilliam, J.P.). Actions of glutamate and kainic acid on the lobster muscle fibre and the frog spinal cord.

438P ADAMS, P.R. & BROWN, D.A. Actions of γ-aminobutyric acid (GABA) on ganglionic transmission and ganglion cell excitability.

439P DICK, FABIENNE & KELLY, J.S. L-2,4-diaminobutyric acid (L-DABA) as a selective marker for inhibitory nerve terminals in rat brain.

440P MEIER, J. (introduced by Berde, B.). Bioanalytical assay of clozapine and its N-oxide metabolite and the determination of their blood levels in the dog.

441P HEWICK, D.S. & MORELAND, T.A. (introduced by Marshall, P.B.). An NADPH dependent warfarin reductase in human and rat liver and kidney soluble fraction.

442P McKILLOP, D. & POWIS, G. The effect of a single dose of ethanol on the hepatic microsomal metabolism of foreign compounds in the rat.

443P AUTY, R.M. & BRANCH, R.A. (introduced by Bhoola, K.D.). The metabolism of ethyl, n-propyl, n-butyl and iso-amyl alcohols by the isolated perfused rat liver.

444P BENNETT, T. & GARDINER, SHEILA M. (introduced by Birmingham, A.T.). The effects of intravenous injections of vinblastine or vincristine on the responses of the rat heart to nerve stimulation and to drugs.

445P COTTNEY, J. & LEWIS, A.J. Mechanisms of concanavalin A-induced inflammation in the rat.

446P BLOGG, C.E., BRITTAIN, R.T., SIMPSON, B.R. & TYERS, M.B. AH 10407: A novel, short-acting, competitive neuromuscular blocking drug in animals and man.

447P ATTERWILL, C.K., MAHONEY, ANNE & NEAL, M.J. The uptake and subcellular distribution of ^{3}H-choline by the retina.

448P SCHILD, H.O. Drug desensitization and cross-desensitization in guinea-pig ileum.

448P TRIST, D.G. & WEATHERALL, M. The effect of isoprenaline on ^{86}Rb uptake by horse lymphocytes *in vitro.*

449P FOZARD, J.R. & MWALUKO, G.M.P. Stimulant effects of 5-hydroxytryptamine on cardiac sympathetic nerves.

450P GRAHAM, C.W., GREEN, A.R., WOODS, H.F. & YOUDIM, M.B.H. 5-Hydroxytryptamine synthesis in the isolated perfused rat brain.

451P LIVINGSTON, A. & PHILLIPS, ELIZABETH. Distribution of chlorpromazine and its metabolites in subfractions of rat brain.

452P TURNBULL, M.J. & WATKINS, J.W. Change in sensitivity to pentobarbitone and halothane induced by acute administration of central nervous system depressant drugs.

453P MOORE, K.E. & PHILLIPSON, O.T. (introduced by Beart, P.M.). Effects of dexamethasone on phenylethanolamine N-methyl-transferase (PNMT) and adrenaline (A) in the brains of adult and neonatal rats.

454P BRYANT, B.J., McCULLOCH, M.W., RAND, M.J. & STORY, D.F. Release of ^{3}H-(−)-noradrenaline from guinea-pig hypothalamic slices: effects of adrenoceptor agonists and antagonists.
455P DARGIE, H.J., DOLLERY, C.T. & LEWIS, P.J. Prevention of DOCA saline hypertension by central 6-hydroxydopamine: role of saline intake.
455P HIRSCH, J.C., TASSIN, J.P. & THIERRY, A.M. Loss of noradrenergic and dopaminergic terminals in the chronically isolated cerebral cortex of the cat.
456P ELKHAWAD, A.O., MUNDAY, K.A., POAT, JUDITH A. & WOODRUFF, G.N. The effect of dopamine receptor stimulants on locomotor activity and cyclic AMP levels in the rat striatum.
457P MILLER, R. (introduced by Iversen, L.L.). Inhibition of dopamine-sensitive adenylate cyclase in rat basal ganglia and other hormone sensitive adenylate cyclase systems by neuroleptic drugs.
458P CALLINGHAM, B.A. & LYLES, G.A. Some effects of age upon irreversible inhibition of cardiac MAO.
459P BEVAN, P., BRADSHAW, C.M. & SZABADI, E. Tricyclic antidepressants and monoamines: the relationship between uptake blockade and potentiation of neuronal responses.
460P AHTEE, LIISA. Effect of drugs on metoclopramide-induced catalepsy and increase in striatal homovanillic acid content.
461P BRIMBLECOMBE, R.W., BUXTON, D.A. & REDFERN, P.H. Drug effects on avoidance behaviour in selected strains of rats.
462P BRADLEY, P.B. & BRAMWELL, G.J. A stereospecific action of morphine on brain stem neuronal activity: a microiontophoretic study.
463P BENOY, CHRISTINE J., EL FELLAH, M.S., SCHNEIDER, R. & WADE, O.L. Tolerance to sympathomimetic bronchodilators in the guinea pig lungs.
464P JONES, R.L. Actions of prostaglandins on the arterial system of the sheep: some structure-activity relationships.
465P HERMAN, A.G. & MONCADA, S. Release of prostaglandins and incapacitation after injection of endotoxin in the knee joint of the dog.
466P PARRATT, J.R. & STURGESS, R.M. The protective effect of sodium meclofenamate in experimental endotoxin shock.
467P CRUTCHLEY, D.J. & PIPER, PRISCILLA J. Inhibition of the pulmonary inactivation of prostaglandins in rabbit *in vivo*.
467P BLACKWELL, G.J., FLOWER, R.J., PARSONS, M.F. & VANE, J.R. Factors influencing the turnover of prostaglandin synthetase.
469P PUGH, D.M., SHARMA, S.C. & WILSON, C.W.M. Inhibitory effect of L-ascorbic acid on the yield of prostaglandin F from the guinea-pig uterine homogenates.
470P GREAVES, M.W., KINGSTON, W.P. & PRETTY, KATHLEEN. Action of a series of non-steroid and steroid anti-inflammatory drugs on prostaglandin synthesis by the microsomal fraction of rat skin.

Br. J. Pharmac. (1975), 53, 317-322

CHOLINERGIC MECHANISMS IN CENTRAL THERMOREGULATION IN PIGEONS

NARESH CHAWLA, M.B.L. JOHRI, P.N. SAXENA & K.C. SINGHAL
Department of Pharmacology, J.N. Medical College, A.M.U., Aligarh, India

1 In unanaesthetized pigeons the effect on cloacal temperature was studied of acetylcholine (ACh), carbachol, atropine and (+)-tubocurarine injected into a cannulated lateral cerebral ventricle. The experiments were carried out at an ambient temperature of 19-25° C.

2 ACh or carbachol injected intraventricularly produced hyperthermia, and in larger doses hyperthermia followed by hypothermia. These were central effects because they were not obtained when these drugs were injected in the same doses intravenously.

3 Atropine injected intraventricularly produced hypothermia which was greater and longer lasting than the hypothermia produced with the same dose of atropine injected intravenously. After the intraventricular injection of atropine the hyperthermic effects of ACh and of carbachol were abolished.

4 (+)-Tubocurarine injected intraventricularly produced a long-lasting hyperthermia in doses which had no effect on temperature when injected intravenously. After the intraventricular injection of tubocurarine the hypothermic effects of ACh and of carbachol were abolished.

5 It is concluded that the effects of ACh and carbachol imitate the effects of ACh released from cholinergic neurones in the central pathway involved in temperature regulation. The hypothermic effect of atropine is attributed to unmasking the activity of continuously released ACh acting on nicotinic receptors, and the hyperthermic effect of tubocurarine to unmasking the activity of continuously released ACh acting on muscarinic receptors.

Introduction

Burn & Dutta (1948) were the first to suggest involvement of cholinergic mechanisms in temperature regulation because atropine lowered body temperature in mice. Since then abundant evidence has been obtained in support of this suggestion in a number of mammals by studying the effects of cholinomimetic substances on body temperature following their injection into the cerebral ventricles or into different regions of the hypothalamus. Experiments of this kind were done in mice (Friedman & Jaffe, 1969), rats (Hulst & de Wied, 1967; Meeter & Wolthuis, 1968; Myers & Yaksh, 1968; Lomax, Foster & Kirkpatrick, 1969; Meeter, 1969, 1971; Avery, 1972; Baird & Lang, 1973), sheep, goats and rabbits (Bligh, Cottle & Maskrey, 1971), cats (Baird & Lang, 1973) and monkeys (Myers & Yaksh, 1969). The nature of the thermoregulatory response to cholinomimetic substances was found to differ in different species and to be dependent on the dose of the substance injected, the ambient temperature, and when injected into the hypothalamus on the actual site of injection.

Evidence that in birds cholinergic neurones are involved in central control of body temperature is provided by the results obtained in the present experiments. In these the temperature effects of cholinomimetic substances were examined when injected into a cannulated lateral cerebral ventricle of pigeon before and after atropine or (+)-tubocurarine similarly injected.

Methods

Healthy pigeons of either sex and weighing 200-300 g were used. Food and water were withheld during the period of the experiment. Room temperature varied between 19-25° C.

The methods of implanting a cannula into the right lateral ventricle under intravenous pentobarbitone sodium (30 mg/kg) anaesthesia, of injecting drugs through this cannula, and of verifying its correct placement were the same as described elsewhere (Chawla, Johri, Saxena & Singhal, 1974). Experiments were started not earlier than 2-3 days after the operation when the birds had recovered; injections were made on the same bird not more frequently than once every second day.

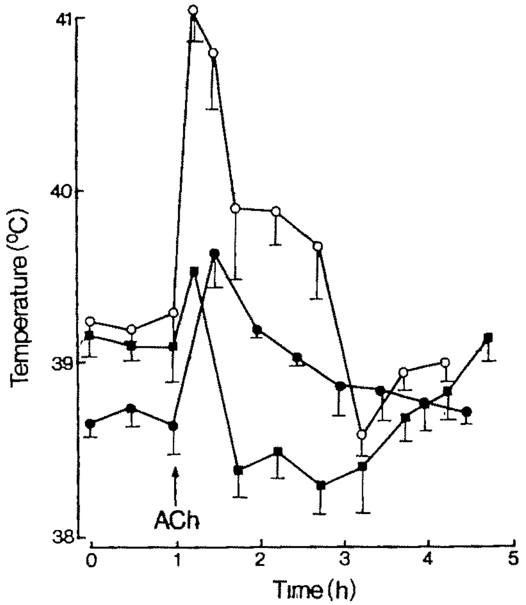

Figure 1 Records of mean cloacal temperature obtained from unanaesthetized pigeons. At the arrow injection into a lateral ventricle of 100 μg acetylcholine (ACh) (●), 200 μg ACh (○) and 200 μg ACh plus 100 μg physostigmine (■). The vertical bars (shown in one direction only) indicate s.e. mean of six experiments

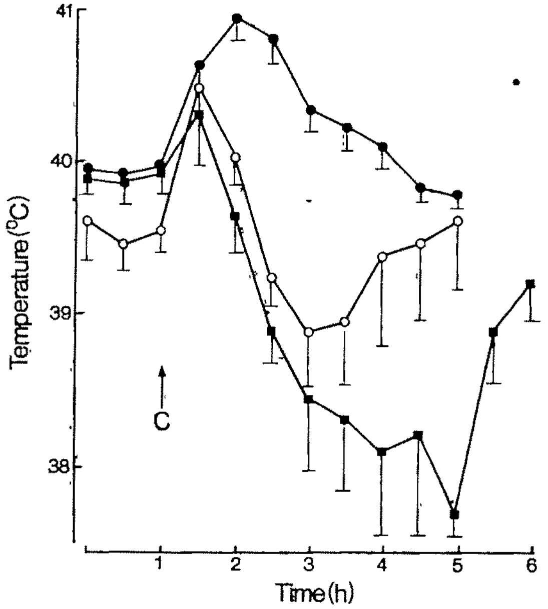

Figure 2 Records of mean cloacal temperature obtained from unanaesthetized pigeons. At the arrow injection into a lateral cerebral ventricle of 2.5 μg (●), 5 μg (○) and 7.5 μg (■) of carbachol (C). The vertical bars (shown in one direction only) indicate s.e. mean of six experiments

Cloacal temperature was taken with a thermistor probe inserted about 2 cm deep into the cloaca, held in position with a piece of tape and connected to a multichannel telethermometer. Readings were taken every 6 min at the beginning and every 15 min later on in the experiment. The temperature records of Figures 1-4 were plotted from these readings.

The drugs used were acetylcholine chloride (ACh), physostigmine sulphate, carbamylcholine chloride (carbachol), atropine sulphate and (+)-tubocurarine chloride. All doses given in the text refer to the salts. The drugs were dissolved in pyrogen-free distilled water. For injections into the lateral ventricle the volume was always 0.02 ml whatever dose was tested. Injections of 0.02 ml distilled water served as controls. For intravenous injections into the pectoral vein the drugs were injected in a volume of 0.1 ml. Injections were made after the cloacal temperature had been relatively stable for at least one hour.

Results

Apart from a transient rise which occurred in some pigeons as a result of excitement due to handling of the birds, the cloacal temperature was not affected by control injections of distilled water into the cerebral ventricle or into the pectoral vein.

Cholinomimetic substances

Intravenous injections of ACh and carbachol produced dose-dependent hypothermias. The mean fall produced in six experiments with each drug was 0.4 ± 0.12°C for 200 μg of ACh and 0.6 ± 0.15°C for 7.5 μg of carbachol.

Intraventricular injections of ACh alone or of ACh with physostigmine produced changes in cloacal temperature, restlessness and mild convulsions which lasted for a few minutes. With the smaller doses of ACh the sole effect on temperature was a rise, with the larger doses the rise was followed by a fall and a biphasic response was obtained. When the ACh was injected together with physostigmine the initial rise in temperature was cut short by a steep fall which dominated the picture. These temperature effects are illustrated in Figure 1 which gives the mean changes obtained with six injections of either 100 or 200 μg of ACh or of 200 μg ACh together with 100 μg of physostigmine. With 100 μg ACh, the temperature

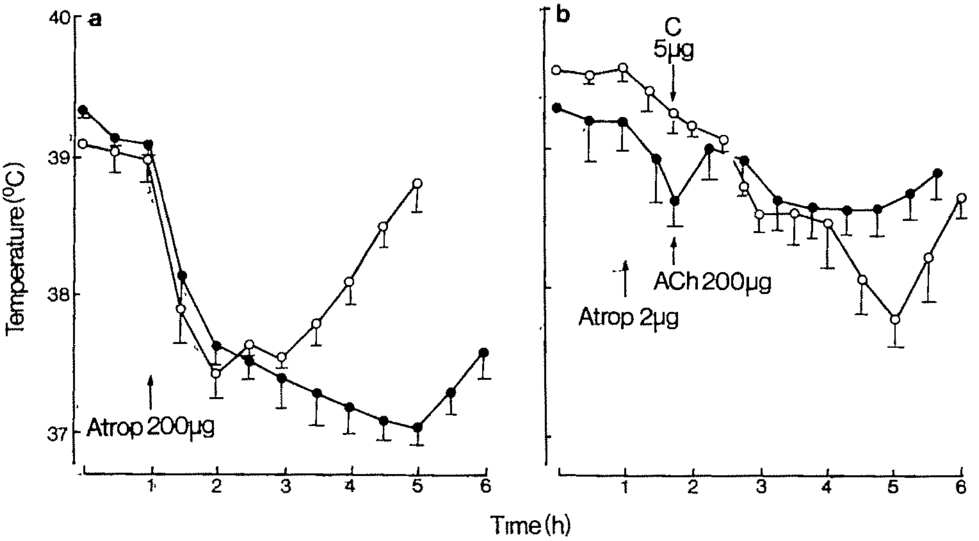

Figure 3 Records of mean cloacal temperature obtained from unanaesthetized pigeons. (a) At the arrow injection of 200 µg atropine (Atrop) into a lateral cerebral ventricle (●) or intravenously (○). (b) At the first arrow injection of 2 µg atropine (Atrop) into a lateral cerebral ventricle. At the second arrow, 45 min later, injection of 200 µg acetylcholine (ACh) or 5 µg carbachol (C) into a lateral cerebral ventricle. The vertical bars (shown in one direction only) indicate s.e. mean of six experiments in (a) and of eight ACh or 10 carbachol experiments in (b).

rose by 1°C, with 200 µg ACh it rose by 1.75° C, and the rise was followed after about 2 h by a fall to 0.7° C below the pre-injection level. When 200 µg ACh was injected together with 100 µg physostigmine, the initial rise of temperature amounted to only 0.35° C; it was followed within about 30 min by a steep fall to 0.9° C below the pre-injection level and temperature then remained at this level for about 1.5 hours.

The changes in cloacal temperature following intraventricular injections of carbachol were similar to those produced by ACh or ACh together with physostigmine. In addition, there occurred excitement, miosis, salivation lasting 20-30 min and in some birds defaecation. Figure 2 illustrates the mean changes in cloacal temperature produced by six injections of either 2.5, 5, or 7.5 µg carbachol. With 2.5 µg the response consisted of a rise of 1° C; with 5 µg, the temperature rose initially more rapidly than with 2.5 µg; but in less than 30 min when the temperature had risen 0.9° C, it began to fall and fell during the following 80 min to 1.1° C below the pre-injection level. With 7.5 µg the initial rise in temperature during the first 30 min after the injection was only 0.35° C; the temperature then began to fall and continued to do so during the following 4 h when it had fallen to 2.2° C below the pre-injection level.

Atropine

Injected intravenously or intraventricularly in doses between 2 and 200 µg atropine produced a dose-dependent fall in cloacal temperature. The only difference in the effects produced with the two routes of administration was that on intraventricular injection the fall was greater and more prolonged. This is seen for 200 µg atropine in the two records of Figure 3a.

It was further found that on intraventricular injection, a dose of 2 µg atropine was sufficient to prevent the hyperthermic effect of a subsequent intraventricular injection of either ACh or carbachol. This is illustrated by Figure 3b. The two records show that 2 µg of atropine which itself produced a small fall in temperature, prevented the rise usually produced by an intraventricular injection of 200 µg ACh or of 5 µg of carbachol, which were injected 45 min after the atropine. It is difficult to be certain whether the atropine also prevented the late hypothermic effect produced by intraventricular injections of ACh and carbachol since the atropine injection itself produced a fall in temperature. From the prolonged large fall seen in Figure 3b when the intraventricular injection of 2 µg of atropine was followed by one of carbachol it would appear that

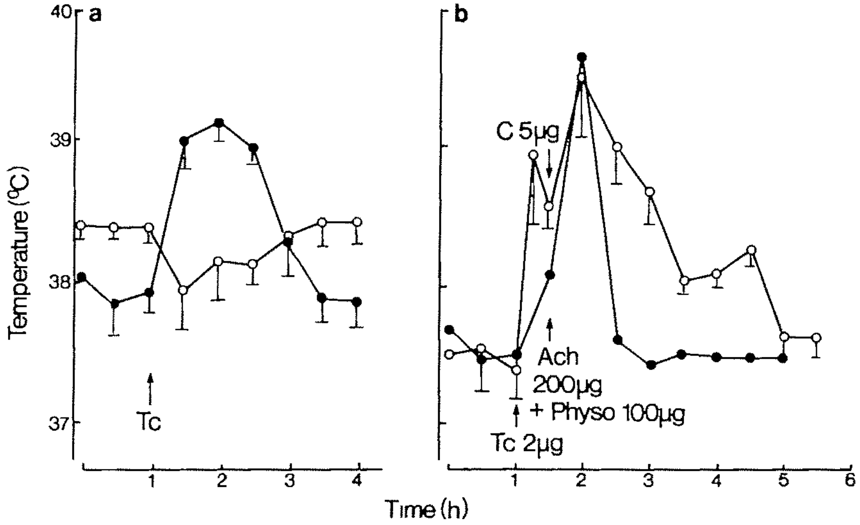

Figure 4 Records of mean cloacal temperature obtained from unanaesthetized pigeons. (a) At the arrow injection of 2 μg (+)-tubocurarine (Tc) into a lateral cerebral ventricle (●) and of 8 μg (+)-tubocurarine intravenously (○). (b) At the first arrow injection of 2 μg (+)-tubocurarine into a lateral ventricle. At the second arrow, 30 min later, injection of 200 μg acetylcholine (ACh) plus 100 μg physostigmine (Physo) or of 5 μg carbachol (C) into a lateral cerebral ventricle. The vertical bars (shown in one direction only) indicate the s.e. mean of six experiments in (a), and of six ACh or eight carbachol experiments in (b).

the hypothermic effect of atropine had summed with that of carbachol.

(+)-Tubocurarine

Injected intravenously, 2 μg of (+)-tubocurarine had no effect on cloacal temperature and 8 μg produced a slight fall as illustrated in Figure 4a. Injected intraventricularly, tubocurarine produced convulsions and a rise in temperature. With 2 μg, short-lasting bouts of tonic-clonic convulsions occurred in many but not in all birds during the first 5-10 min after the injection; temperature rose regularly, whether convulsions occurred or not, and remained high long after the convulsions had come to an end. Figure 4a shows for six birds the mean rise of 1.2° C lasting about 2.5 hours. Intraventricular injections of 8 μg of (+)-tubocurarine were regularly followed by strong tonic-clonic convulsions which resulted in death within a few minutes.

The effect of an intraventricular injection of 2 μg of (+)-tubocurarine on an intraventricular injection, given 30 min later, of ACh with physostigmine, or of carbachol is illustrated in Figure 4b. The rise in temperature produced by the two injections following each other was greater than that produced by either injection alone. This suggests that (+)-tubocurarine, unlike atropine, does not prevent the hyperthermic effect produced either by ACh injected together with physostigmine or by carbachol. On the other hand, the late hypothermic response which these substances produced and which appeared not to be affected by atropine was abolished by (+)-tubocurarine.

Discussion

The cholinomimetic substances, ACh and carbachol, were found to produce hyperthermia when injected into the cerebral ventricles of pigeons, and in larger doses the hyperthermia was followed by hypothermia. These are central effects because they were not produced when the substances were injected intravenously. The action of ACh on the thermoregulatory system of pigeons appears to be similar to that in cats and monkeys, but different from that in rats.

From the results obtained with atropine and tubocurarine it is evident that different receptors are involved. The hyperthermia can be attributed to activation of muscarinic receptors because it was prevented by atropine. Since atropine did not seem to affect the hypothermic response it is probably not due to activation of muscarinic receptors. The fact that it was prevented by (+)-tubocurarine would suggest that it is due to activation of nicotinic receptors. However, this conclusion depends on the assumption that (+)-tubocurarine inhibits nicotinic receptors in the central nervous system in the same way as in the peripheral nervous system, i.e. at the motor endplates or the synapses of autonomic ganglia.

On the central nervous system (+)-tubocurarine exerts an excitatory strychnine-like action due to depolarization of the central synapse. This action does not manifest itself on intravenous administration because (+)-tubocurarine does not readily pass the blood-brain barrier. The depolarizing effect was first described by Chang (1953) who applied (+)-tubocurarine topically to the cerebral cortex of cats and rabbits. The effect has been confirmed by many authors who introduced tubocurarine by micro-injection into the grey matter of the cerebral cortex or into the hippocampus (for references see Banerjee, Feldberg & Georgiev, 1970). The depolarization is thought to be due, at least in part, to 'disinhibition', i.e., to depression of inhibitory potentials. The tonic-clonic convulsions observed in pigeons after intraventricular injections of tubocurarine can be attributed to an excitatory action on the hippocampus because they also occur in cats when tubocurarine is injected intraventricularly, and in this species were shown to result from an excitatory action on the hippocampus reached from the posterior half of the lateral ventricle (Feldberg & Fleischhauer, 1963). In cats tubocurarine applied intraventricularly produces in addition shivering due to an excitatory action on the hypothalamus which is reached from the third ventricle (Carmichael, Feldberg & Fleischhauer, 1962). Both convulsions and shivering would raise body temperature, so the rise in temperature produced in pigeons could be due to an excitatory action on these two structures. However, temperature remained elevated for over 2 h whereas the convulsions came to an end after a few minutes and shivering was not observed. It is difficult therefore, to attribute the hyperthermia produced by (+)-tubocurarine as well as the prevention of the late hypothermia produced by acetylcholine and carbachol to a remnant of the excitatory action of (+)-tubocurarine on the hippocampus and hypothalamus. Instead they could well be due to blocking of nicotinic receptors on the assumption that tubocurarine exerts such an action on the central synapse in addition to its depolarizing effect.

Both muscarinic and nicotinic receptors have been postulated to explain the effects on body temperature obtained with acetylcholine injected into the cerebral ventricles or introduced by microinfusion into different parts of the hypothalamus. Its hyperthermic effect is thought to be due to an action on muscarinic, its hypothermic effect to an action on nicotinic receptors. In monkeys, Myers & Yaksh (1969) found that microinfusions of ACh as well as of carbachol into various regions throughout the hypothalamus produced hyperthermia, but microinfusion into one circumscribed region at the caudal border of the posterior hypothalamus produced hypothermia. This would localize the muscarinic receptors throughout the hypothalamus and the nicotinic receptors to its posterior portion. Nicotine itself produces hypothermia when perfused in monkeys through the cerebral ventricles (Hall & Myers, 1971a) but on microinfusion into the hypothalamus, hyperthermia from the posterior, and hypothermia from the anterior hypothalamus (Hall & Myers, 1971b). To explain these two effects as being due to actions on nicotinic receptors one would have to postulate inhibitory and excitatory nicotinic receptors. Baird & Lang (1973) obtained in cats hyper- and hypothermic responses to ACh and metacholine and hypothermic responses to nicotine when these drugs were injected intraventricularly. The hyperthermic responses were prevented by atropine and the hypothermic responses by mecamylamine. They therefore postulated muscarinic receptors to account for the hyper-, and nicotinic receptors to account for the hypothermic responses.

The fact that in pigeons the hyperthermic effect was obtained with smaller doses of ACh or carbachol than the hypothermia which, when it occurred, followed the hyperthermia, is explained on the assumption that the sites of the muscarinic receptors are more readily accessible on penetration from the ventricular lumen than the site of the nicotinic receptors. The possibility of a difference in accessibility of the two receptor sites from the ventricular lumen was first envisaged by Hall & Myers (1971a).

The finding that atropine injected intraventricularly into pigeons produced a fall in cloacal temperature can be explained by its blockade of a muscarinic action of continuously released acetylcholine, whereas the rise produced by (+)-tubocurarine, if not due to its depolarizing action, could be explained by blockade of a nicotinic action of released acetylcholine. A tonic activity in the cholinergic neurones subserving the thermoregulatory system has also been observed in cats and rats. In cats, Baird & Lang (1973) obtained hypothermia on intraventricular injection of atropine and in rats, in which the main thermoregulatory effect of acetylcholine is hypothermia, atropine raises temperature (Kirkpatrick & Lomax, 1967).

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NORADRENALINE AND MOTOR TRANSMISSION IN THE VAS DEFERENS OF THE MOUSE

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1 A comprehensive investigation of the innervation of the vas deferens of the mouse was made using pharmacological, histochemical and electronmicroscopical techniques.

2 Guanethidine inhibited the response of the vas to transmural stimulation and potentiated the response to noradrenaline (NA). Phentolamine abolished responses to NA and to transmural stimulation.

3 After chemical sympathectomy degenerative changes were seen in presumptive noradrenergic axons; histochemical fluorescence due to catecholamines was absent. The vas failed to respond to transmural stimulation, and a 10-fold increase in sensitivity of the vas to exogenous NA was observed.

4 NA is shown to diffuse slowly through this tissue whose muscle cells are densely packed. This is discussed in relation to the apparent 'insensitivity' of the vas to exogenous NA.

5 A cholinergic component was identified histochemically which did not contribute significantly to the motor response of the vas as chemical sympathectomy abolished completely the motor response elicited by transmural stimulation.

6 It is concluded that NA is the motor transmitter for the smooth muscle of the vas deferens of the mouse.

Introduction

The vasa of rat, guinea-pig and mouse have a high content of noradrenaline (NA) (Sjöstrand, 1965) and have been shown to contain presumptive noradrenergic neurones (Richardson, 1962; Burnstock & Robinson, 1967; Yamauchi & Burnstock, 1969). However, relatively high concentrations of exogenous NA are required to induce contractions of vasa of guinea-pig (Birmingham & Wilson, 1963; Ambache & Zar, 1971) rat (Graham, Al Katib & Spriggs, 1968; Birmingham, 1970) and mouse (Holman, 1970). The difficulty in obtaining responses to exogenous NA of equal magnitude to those of nerve stimulation and the ability of NA to inhibit responses evoked by nerve stimulation has led to Ambache & Zar (1971) challenging the concept that NA acts as the motor transmitter substance in the vas of guinea-pig. In addition, α-adrenoceptor blocking drugs have been shown to block the effects of exogenous NA without affecting responses to nerve stimulation (Bentley & Smith, 1967; Ambache & Zar, 1971).

The present study was undertaken to establish the neurotransmitter responsible for the motor response of the longitudinal muscle of the vas deferens of mouse by the use of histological, histochemical and pharmacological techniques.

Methods

Mature male albino mice, T.O. strain, weighing 25-35 g and over four weeks old were injected intravenously with either 6-hydroxydopamine (6-OHDA) 1 mmol/kg or vehicle. The vehicle for 6-OHDA was 0.2 mg ascorbic acid/ml 0.9% w/v NaCl solution (saline). Twenty-four hours later the mice were killed by cervical dislocation and both vasa deferentia removed.

Single organs were suspended in Huković's solution (1961), gassed with 5% CO_2 in O_2 at 32° C. The tissue was subjected to 300 mg applied tension and allowed to equilibrate for 30-45 minutes. Isometric contractions were monitored on a Devices two-channel pen recorder via a 2 oz dynamometer UFI strain gauge. Transmural stimulation (Birmingham & Wilson, 1963) of vas suspended between two parallel platinum wire electrodes and immersed in a 50 ml bath was by means of an SRI 6053 stimulator delivering rectangular pulses of 0.3 ms duration at frequencies of 20 Hz and of supramaximal voltage every 3.25 minutes. An oscilloscope was used to confirm the uniformity and reproducibility of the stimulation parameters under experimental conditions with the electrodes totally immersed in the bathing fluid.

In experiments not involving transmural stimulation, tissues were suspended in 10 ml baths. NA or acetylcholine (ACh) was left in contact with the tissue for 30 s every three minutes. Bathing fluid was replaced at 1.5 min intervals.

An analysis of covariance (Snedecor, 1956) was undertaken to ascertain whether the regression lines of log dose-response curves for NA or ACh in normal vasa were parallel or coincident to those obtained in 6-OHDA pretreated vasa.

Catecholamine histofluorescence

Cryostat-cut 18 μm sections of frozen vasa were dried and exposed to moist formaldehyde vapour under strictly controlled conditions, after the method of Spriggs, Lever, Rees & Graham (1966). The sections were viewed with a Zeiss fluorescence microscope.

Electron Microscopy

Routine investigation. Approximately 2 mm of tissue were removed from the epididymal end of the vas and cut into 1 mm cubes of tissue which were immersed in 1% osmium tetroxide buffered to pH 7.5 with veronal acetate for 1 h at 4° C, dehydrated and subsequently embedded in araldite. Fine sections were stained with lead citrate (Reynolds, 1963) and viewed with a Phillips 300 electron microscope.

Demonstration of acetylcholinesterase (AChE). Specimens were fixed in glutaraldehyde and incubated for 4 h at pH 5.5 with acetylthiocholine in the presence of 2×10^{-4} M ethopropazine (a pseudocholinesterase inhibitor) after the method of Lewis & Shute (1966). If physostigmine, a non-specific anticholinesterase, was included in the incubating medium instead of ethopropazine no AChE reaction product was seen in the tissue.

Extracellular diffusion of [^{3}H]-noradrenaline or [^{14}C]-sorbitol

Male mice used in [^{3}H]-noradrenaline determinations were pretreated with phenoxybenzamine (1.2×10^{-4} mol/kg intravenously) 30 min before they were killed (Gillespie, Hamilton & Hosie, 1970) and the vasa excised. Mice used for the D-sorbitol-[1-^{14}C] determinations received no pretreatment.

Incubation media

The stripped vasa were placed immediately in ice cold Krebs-Ringer bicarbonate buffer, pH 7.4 (Sachs, 1970) for [^{3}H]-noradrenaline determination ([^{3}H]-NA, specific activity = 7.3 Ci/mmol) and unlabelled noradrenaline was added to the buffer to make a final concentration of 1.22×10^{-4} M. Desmethylimipramine (DMI) (9.4×10^{-7} M) and ascorbic acid (0.2 mg/ml) were included in the incubating medium. For D-sorbitol-[1-^{14}C] determination unlabelled sorbitol was added to the incubating buffer together with D-sorbitol-[1-^{14}C] (specific activity = 3 mCi/mmol) in order to obtain a concentration of 2.7×10^{-6} M (Morgan, Henderson, Regan & Park, 1961).

In vitro *incubation*

Two vasa were placed in each test tube containing 1 ml of incubation medium and incubated in a metabolic shaker at 32° C. The medium was pre-gassed with 5% CO_2 in O_2; each test tube was stoppered with a tight fitting rubber bung and re-gassed for 10 s after 30 min incubation (Sachs, 1970). Vasa were removed after 0.5, 1, 5, 10, 30 and 60 min from the incubation medium.

Determination of total radioactivity by liquid scintillation

One vas (approx. 20 mg) was placed in 1 ml digest medium (2N methanolic potassium hydroxide; Petroff, Patt & Nair, 1965) and placed in an oven at 60° C overnight. Formic acid (0.1 ml) was added to 10 ml of scintillation medium (toluene, 700 ml; ethoxyethanol, 300 ml; PPO, 4 g; POPOP, 0.1 g; Hall & Cocking, 1965) before 0.2 ml aliquots of sample were added for counting.

The total radioactivity was measured in a Unilux II (Nuclear-Chicago), liquid scintillation counter. All counts were corrected for errors due to quenching and counting efficiency, and are expressed per mg wet weight of vas.

Drugs used were: acetylcholine chloride (Sigma), acetylthiocholine chloride (Sigma), desmethylimipramine hydrochloride (Geigy), ethopropazine hydrochloride (May & Baker), guanethidine sulphate (Ciba), 6-hydroxydopamine hydrochloride (Ralph N. Emanuel), (−)-noradrenaline bitartrate (Koch-Light), (±)-[^{3}H]-noradrenaline hydrochloride (7.3 Ci/mmol) (Radiochemical Centre, Amersham, England), phenoxybenzamine hydrochloride (S.K.F.), phentolamine mesylate

(Ciba), physostigmine salicylate (B.D.H.), sorbitol (B.D.H.), D-sorbitol-[1-^{14}C] (3mCi/mmol) (Radiochemical Centre, Amersham, England). Doses refer to the base.

Results

Responses of normal vasa to noradrenaline, acetylcholine or transmural stimulation

The vas responded to NA with an initial rapid contraction which reached a maximum usually within 5 seconds The relationship between the tension developed in the vas and the log concentration of NA in the bath was linear for the dose range 1.2×10^{-7} M to 1.2×10^{-4} M (correlation coefficient, $r = 0.93$; $P < 0.001$) (Figure 1). The mean maximum tension developed in response to NA in 12 vasa was 0.90 ± 0.28 g which was 62.5% of the mean maximum tension developed during transmural stimulation (1.44 ± 0.08 g).

Responses to ACh, although often irregular and exhibiting several peaks of tension, showed a linear relationship to the log bath concentration of ACh in the range 1×10^{-6} to 1×10^{-3}M ($r = 0.92$; $P < 0.001$) (Figure 2). The mean maximum tension induced by ACh in eight vasa was 0.48 ± 0.040 g, equivalent to 33.7% of the mean maximum tension produced in response to transmural stimulation.

The vas contracted in response to transmural stimulation with 0.3 ms rectangular pulses of supramaximal voltage applied at 20 Hz for 10 s every 3.25 minutes. In the presence of guanethidine (2.0×10^{-6} M) the response of the vas to transmural stimulation was abolished, that to NA (6×10^{-6} M) potentiated and that to ACh (2×10^{-4} M) unmodified. Phentolamine (10^{-7} M) inhibited the response of the vas to NA and in higher concentration (5.3×10^{-5} M) abolished completely responses to NA and to transmural stimulation. Responses to ACh were unaffected by phentolamine.

Vasa from 6-hydroxydopamine pretreated mice

Transmural stimulation with 0.3 ms pulses failed to elicit contractions from the vasa of 6-OHDA-treated mice. With maximum voltage from the stimulator (13 V across immersed electrodes) the mean increase in tension from six denervated vasa was 1.2 ± 0.8% of that elicited from control vasa ($P < 0.05$).

The log dose-response curve for NA in 6-OHDA pretreated mice was linear in the dose range 3×10^{-8} M to 6×10^{-6} M ($r = 0.88$; $P < 0.001$) and the slope was not significantly different ($P > 0.05$) from that obtained in vasa from untreated animals. The shift to the left of the curve for NA in treated vasa was significant ($P < 0.01$) (Figure 1).

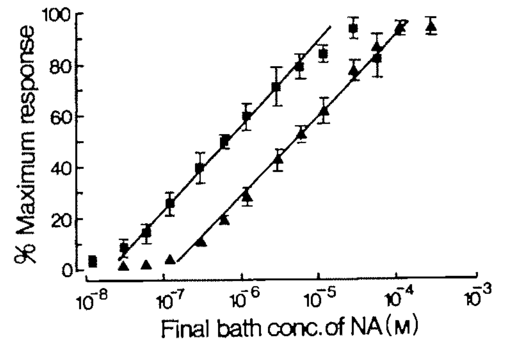

Figure 1 Log dose-response curves for noradrenaline (NA) in normal (▲) and chemically sympathectomized (■) vasa of mouse. Each point is the mean from at least nine vasa. Vertical bars show s.e. mean. The lines are drawn through co-ordinates calculated by the method of least squares analysis in the dose range 1.2×10^{-7} M to 1.2×10^{-4} M for NA in normal vasa and 3×10^{-8} M to 1.2×10^{-5} M for NA in chemically sympathectomized vasa.

A linear log dose-response curve was obtained for ACh in chemically sympathectomized vasa in the dose range 1×10^{-7} M to 1×10^{-3} M ($r = 0.94$; $P < 0.001$) which was neither parallel nor coincident to the regression line for ACh in untreated vasa ($P < 0.01$) (Figure 2). The chemically sympathectomized vasa did respond to lower doses of ACh (10^{-7} M). However, a similar maximal response was attained in both sympathectomized and untreated vasa with 10^{-3} M ACh (Figure 2).

The response of the vas to NA or ACh was qualitatively changed after 6-OHDA pretreatment of the mice. The response to NA reached several peaks of tension during the period of drug contact. The response to ACh was more prolonged in denervated vas preparations than in untreated vasa.

Electron Microscopy

The muscle cells in the mouse vas deferens occur in tightly-packed bundles, the interval between adjacent sarcolemmae being in the order of 100 nm. Large bundles of unmyelinated axons (8.3 ± 0.6 axons per bundle) embedded in Schwann cell processes were found in the extracellular spaces between muscle bundles. Smaller bundles of axons (5.6 ± 0.2 axons) were found in the extracellular spaces within muscle

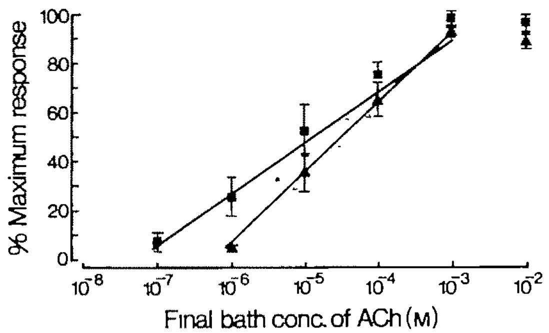

Figure 2 Log dose-response curves for acetylcholine (ACh) in normal (▲) and chemically sympathectomized (■) vasa of mouse. Each point is the mean from eight vasa. Vertical bars show s.e. mean. The lines are drawn through co-ordinates calculated by the method of least squares analysis in the dose range 1×10^{-6} to 1×10^{-3} M for ACh in normal vasa and 1×10^{-7} to 1×10^{-3} M for ACh in chemically sympathectomized vasa.

bundles. The Schwann cell sheath around the axons of the smaller axon bundles was often incomplete, leaving the axolemmae naked in parts.

The number of axons occurring singly and in bundles of two or three axons exceeded the number of muscle cells in any one plane of section. A mean of 82 axons were observed in a 7225 μm^2 grid space.

Axons partially denuded of Schwann cell investment and occurring in bundles of four or more were situated further from a muscle membrane than axons occurring in bundles of two or three axons. The distribution of the latter was similar to that of axons occurring singly (Figure 3). The proportion of axons totally or partially devoid of Schwann cell processes, situated within 100 nm of a muscle membrane, constitute over 70% of axons occurring singly, 60% of axons in bundles of two or three and under 40% of axons in bundles of four or more (Figure 3)

Ultimately some axons emerge from their Schwann cell sheaths to pass singly into extracellular spaces between muscle cells. These axons exhibit alternate constrictions and dilatations along their lengths. Axons are often embedded in the surface of the muscle cell and occasionally are completely surrounded by muscle cell processes (Figure 4).

The percentage of axons completely surrounded by smooth muscle constituted 1.2% of all axons investigated, 5.8% of the single axons investigated and 8.6% of axons lying less than 20 nm from a muscle membrane. These nerves contained small dense core vesicles and were devoid of Schwann cell processes. The subcellular space between the axon and the muscle membrane was between 10-20 nm. No morphological characteristic of the sarcolemma was apparent in relation to the embedded axon. However, an elongated sac of endoplasmic reticulum was found adjacent to

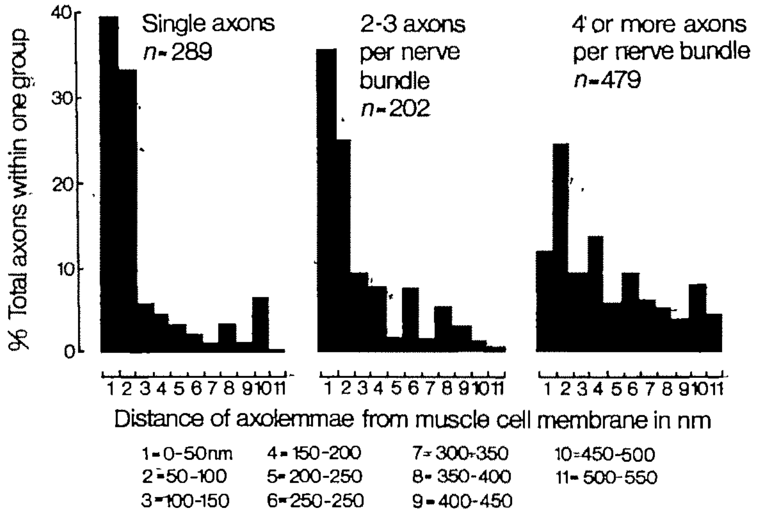

Figure 3 The distribution of axons in the mouse vas deferens. Axons are grouped as occurring singly, in bundles of two to three axons or in bundles of four or more axons and classified according to the distance of their axolemmae from the nearest muscle cell membrane. n = the number of axons investigated per group.

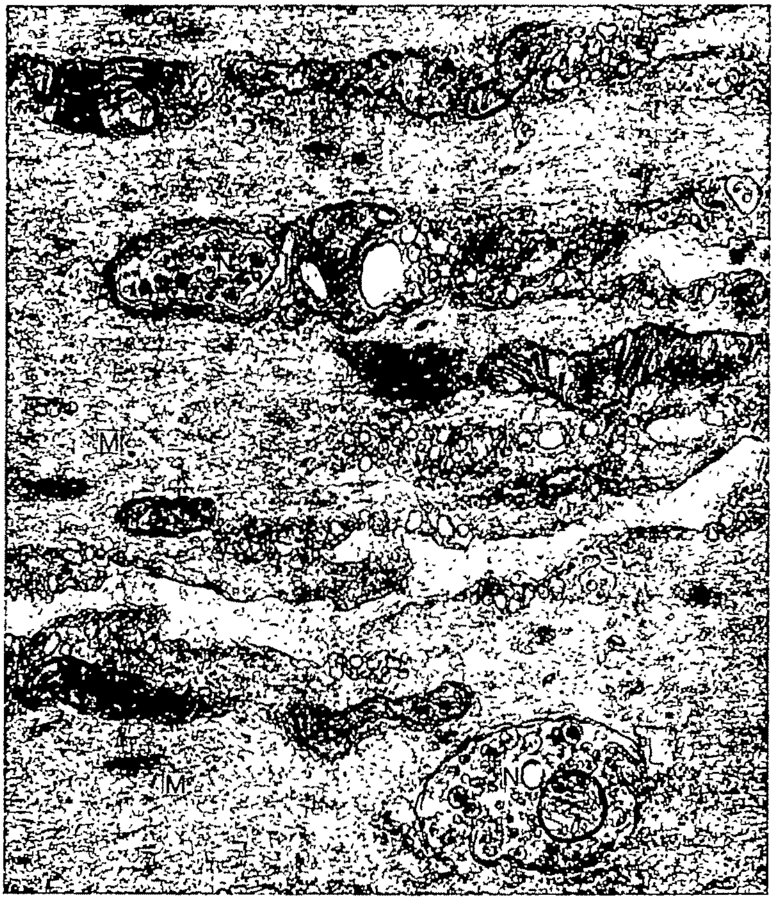

Figure 4 An electronmicrograph illustrating two profiles of noradrenergic axons (N) embedded in the cytoplasm of muscle cells (M) of vas taken from a mouse pretreated 1 h previously with 6-hydroxydopamine 0.2 mmol/kg. The interval separating axolemma from sarcolemma is less than 20 nm. Osmium tetroxide fixation.

the muscle membrane which was found adjacent to 78% of embedded axons. Of the 528 electronmicrographs of osmicated tissue studied, 145 elongated endoplasmic reticular sacs were found adjacent to axons and 200 were observed on muscle membranes remote from axons. These structures were found near 15.6% of single axons, 22.3% of axons in bundles of two or three axons and near 11.5% of axons in bundles of four or more axons.

In glutaraldehyde post-osmicated tissue 85% of axons contained small dense core vesicles (25-60 nm). Of the axon profiles, 22% contained small agranular as well as granular vesicles; 7.5% of axons contained small agranular vesicles only and had AChE reaction product on their axolemmae (Figure 5). In 6-OHDA pretreated vasa the majority of axons (81% of total axons) showed degenerative changes, 5% only having small dense core vesicles (Figure 5). A χ^2 test indicated that there was no significant difference ($P > 0.05$) in the population of AChE positive axons containing agranular vesicles between normal and sympathectomized vasa.

Catecholamine histofluorescence studies

The smooth muscle of the vas was densely innervated with a network of nerves exhibiting the bright green-yellow fluorescence characteristic for catecholamines. Twenty-four hours after an intravenous injection of 6-OHDA no specific fluorescence was observed in the vas.

Extracellular diffusion studies

The diffusion of $[^{14}C]$-sorbitol or $[^{3}H]$-NA into

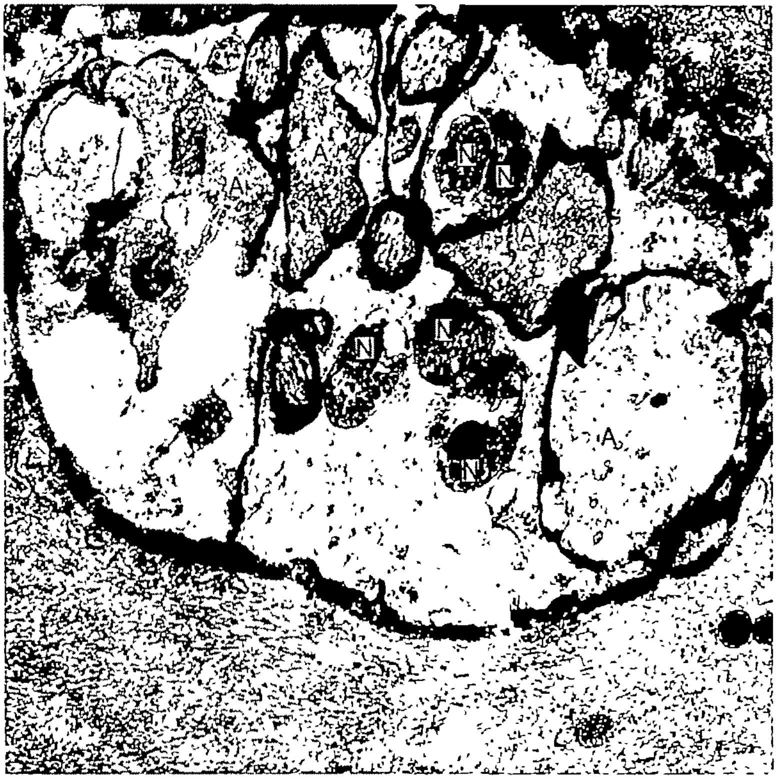

Figure 5 Electronmicrograph showing axons in smooth muscle layer of vas taken from mouse treated 24 h previously with 6-hydroxydopamine 1 mmol/kg, fixed with glutaraldehyde and incubated with acetylthiocholine. Acetylcholinesterase reaction product is seen on the axolemmae of axons which contain small electrontranslucent vesicles (A). These axons are presumptive cholinergic axons. The dense osmophilic masses in axons (N) are characteristic of axonal degeneration.

the extracellular fluid of the vas is expressed as the % accumulation ratio·

$$\frac{\text{d/min } [^{14}\text{C}]\text{-sorbitol (or } [^{3}\text{H}]\text{-NA) per mg vasa}}{\text{d/min } [^{14}\text{C}]\text{-sorbitol (or } [^{3}\text{H}]\text{-NA) per } \mu\text{l medium}} \times 100$$

The accumulation of $[^{14}C]$-sorbitol or $[^{3}H]$-NA by the vas was similar up to 10 min incubation. Between 10 and 60 min the accumulation of $[^{3}H]$-NA exceeded that of $[^{14}C]$-sorbitol (Figure 6).

Discussion

Intravenous administration of 6-OHDA in the mouse resulted in a chemical sympathectomy of the vas deferens. Electron microscopy of these vasa showed presumptive noradrenergic neurones exhibiting degenerative changes similar to those seen after surgical sympathectomy (cf. Van Orden, Bensch, Langer & Trendelenburg, 1967; Lever, Spriggs & Graham, 1968). Histochemical fluorescence due to catecholamines was absent, and the noradrenaline content of the vas was decreased to below 20% of control values (Jones & Spriggs, unpublished observations). Concomitantly neurotransmission was completely suppressed and a ten-fold increase in sensitivity to exogenous NA was observed. These findings together with evidence that guanethidine, an adrenergic neurone blocking drug or phentolamine, an α-adrenoceptor blocking agent, completely block neurotransmission in the vas is sufficient to establish the motor innervation to the vas deferens of the mouse as noradrenergic in nature.

Presumptive cholinergic axons, identified histologically as axons containing agranular vesicles and having an electron dense AChE

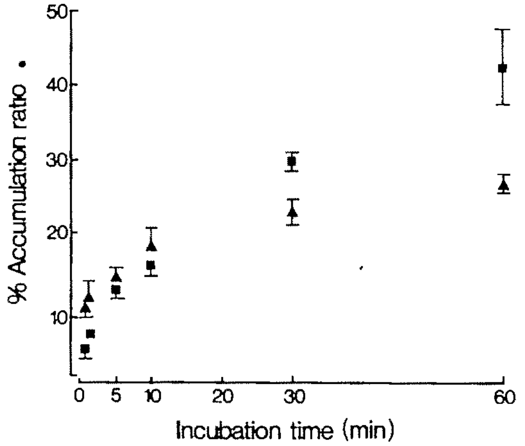

Figure 6 The diffusion of [^{14}C]-sorbitol (▲) or [^{3}H]-noradrenaline (■) through the extracellular spaces of the vas deferens of the mouse. Each point is the mean for eight preparations.

$$\%\ \text{accumulation ratio} = \frac{\text{d/min per mg vasa}}{\text{d/min per }\mu\text{l medium}} \times 100$$

reaction product associated with their axolemmae, constituted less than 10% of the total axon population. In sympathectomized vasa no difference was detected in their appearance or in the number of such axons observed ($P > 0.05$). The contribution of these axons to the functional contraction of the vas is considered negligible as, after chemical sympathectomy with 6-OHDA, the vas failed to respond to nerve stimulation.

The response of the vas to NA and ACh is qualitatively and quantitatively changed by 6-OHDA pretreatment of the mice. The ten-fold increase in sensitivity to NA of the vas of the mouse is similar to the 16-fold increase in sensitivity of the rat vas reported by Birmingham, Paterson & Wojcicki (1970) who used a surgical method for denervation. In agreement also with the work of Birmingham *et al.* (1970) is the increase in sensitivity of the vas to low doses of ACh.

The increase in sensitivity to NA may be attributed to the destruction of the presynaptic neuronal uptake mechanism for noradrenaline following denervation resulting in more NA being available to α-receptors (Birmingham *et al.*, 1970; Ozawa & Sugawara, 1970). However, post-synaptic changes occurring secondarily to denervation cannot be precluded because of the non-specific nature of the supersensitivity and the qualitative changes which also occurred. Cocaine, an inhibitor of uptake$_1$ mechanism, has been shown to be without effect on responses of guinea-pig vasa to ACh or histamine whereas denervation produced a prolonged contraction to each stimulant (Westfall, McClure & Fleming, 1972).

Folkow & Häggendal (1970) estimated that 400 molecules of NA were released per varicosity per impulse assuming that most of tissue NA is present in the vesicles and that the majority of varicosities discharge transmitter when an impulse arrives. This amount of noradrenaline is equivalent to 6×10^{-6} M NA evenly distributed in a junction gap having a size of 1-2 μm^2 and a width of 100 nm. Applying this calculation to the present experiments in which trains of 200 stimuli were used over 10 s, the concentration of NA in the synaptic cleft could reach 1.2×10^{-3} M. We also found that a maximum response of the vas was achieved with a train of 60 stimuli over 2 s, affording a calculated concentration of 7.2×10^{-4} M NA in the synaptic cleft. These estimates presumably err on the high side as no allowances have been made for diffusion or neuronal reuptake of NA; they may, therefore, be considered compatible with the concentration of added NA (1.2×10^{-4} M) which elicited a maximum response of the vas. However, although the vas appears insensitive to NA this does not explain the failure of exogenous NA (even in concentrations up to 1×10^{-3} M) to produce a contraction of similar magnitude to that achieved by transmural stimulation, this may be a consequence of the morphological structure of the vas and the physiological properties of the smooth muscle cells.

The time taken for sorbitol to reach equilibration in tissue is considered to be indicative of the time required for substances to diffuse through the extracellular spaces and Morgan *et al.* (1961) have shown that sorbitol does not penetrate into cardiac muscle cells. In the vas of mouse, sorbitol reaches equilibration in the extracellular fluid between 30 and 60 min after starting incubation. The sorbitol space was 26.8 ± 1.1% after 60 min incubation. This value is comparable with the sorbitol space (22.8% ± 1.1) determined for the atria of mouse (Sachs, 1970). However, equilibration of sorbitol in atrial tissue took between 10 and 30 min incubation in contrast to between 30 and 60 min incubation in vasa. Although the extracellular spaces of both tissues are similar in volume the longer equilibration time for sorbitol in the vas is probably due to the tight packing of the muscle cells in this tissue. The taenia coli of guinea-pig has a larger sorbitol space (40%) which equilibrates faster (5 to 10 min, Goodford & Leach, 1966) than that of vas or atrium of mouse.

In vasa in which both uptake processes for NA into nerve and muscle were inhibited [^{3}H]-NA was accumulated up to 60 min after starting the incubation. The dose of phenoxybenzamine injected into mice 30 min before they were killed has been shown to inhibit the accumulation of NA at all concentrations by smooth muscle, endothelium and nerves of the spleen of cat (Gillespie *et al.*, 1970). DMI has been shown to block the uptake$_1$ mechanism for noradrenaline into nerves (Iversen, 1967).

The persistent accumulation of [^{3}H]-NA after 30 min incubation may indicate that [^{3}H]-NA is taken up by collagen (Gillespie *et al.*, 1970) and other extraneuronal and extramuscular sites whose uptake is not blocked by phenoxybenzamine or DMI. Alternatively, [^{3}H]-NA, having saturated the extracellular spaces may enter nerves and muscles by passive diffusion or via any remaining small fraction of uptake$_1$ and uptake$_2$ mechanisms which remain unblocked.

Full equilibration of the extracellular space of the vas takes 30-60 minutes. The maximum response to noradrenaline (1.2×10^{-4} M) occurs within 30 s of exposure. If the accumulation ratio of noradrenaline in 30 s is expressed as a percentage of the equilibrated sorbitol space then it would appear that in 30 s exogenously administered noradrenaline has diffused through only 15% of the extracellular space, indicating a failure to reach all muscle cell membranes. Furness & Burnstock (1969), using electrophysiological techniques, have reported the inability of the muscle cells of vas of mouse to propagate actively an initiated spike potential although some stimulation may be transmitted passively and in a decremental manner to other cells surrounding the stimulated cell. In contrast to stimulation induced by exogenous NA, transmural stimulation presumably activates all synapses, and every muscle cell in vas of mouse possesses one to four possible neuromuscular synapses, ensuring a maximum contraction of the musculature.

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LACK OF CARDIAC OR BRONCHODILATOR TACHYPHYLAXIS TO ISOPRENALINE IN THE DOG

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1 Tachyphylaxis to heart rate and bronchodilator effects of (±)-isoprenaline was studied in anaesthetized open-chest dogs by 5 procedures.

2 Heart rate responses to a series of intravenous injections of isoprenaline were essentially unchanged before and after a 15 min infusion of isoprenaline (at 3 doses).

3 Heart rate and bronchodilator responses in the same animal to an intravenous injection of isoprenaline were not significantly different before and after a 30 min infusion of isoprenaline (at 2 doses).

4 Heart rate responses were relatively constant to an isoprenaline injection given every 30 min during a 4 h infusion of isoprenaline (at 3 doses), but the magnitude of the response was smallest for the largest infusion dose (highest background heart rate) and greatest for the smallest infusion dose (lowest background heart rate).

5 Heart rate and bronchodilator responses in the same animal to isoprenaline were relatively constant during a 5 h infusion of isoprenaline (at 2 doses).

6 Bronchodilator responses to intratracheally administered isoprenaline aerosol were essentially unchanged during a 4-5 h period using various doses and procedures.

7 Tachyphylaxis to the heart rate or bronchodilator effects of isoprenaline was not observed. The present data give no support to the hypothesis that tachyphylaxis to isoprenaline aerosols is an important mechanism in asthma mortality.

Introduction

Normal subjects have been reported to have a smaller heart rate increase to injected isoprenaline after receiving infusions of small amounts of isoprenaline (Conolly, Davies, Dollery & George, 1972). Kingsley, Littlejohns & Prichard (1972) also studied heart rate increases in normal subjects to isoprenaline and concluded that there was no tachyphylaxis in terms of the dose required to produce a given peak heart rate. Tachyphylaxis to heart rate effects of isoprenaline has also been reported in dogs (Conolly *et al.*, 1971) and cats (Atkinson & Rand, 1968).

Some asthmatic patients were reported to have no bronchodilator response to nebulized isoprenaline (Reisman, Friedman & Arbesman, 1968; Van Metre, 1969; Herxheimer, 1969, 1972a). It is not certain whether this lack of response was due to tachyphylaxis or to other causes (see Discussion). Tachyphylaxis to the bronchodilator effects of isoprenaline was observed in *in vivo* guinea pig experiments by Conolly *et al.* (1971) and Bouhuys, Douglas & Lewis (1972), but not by Pun, McCulloch & Rand (1971).

The purpose of the present investigation was to study the development of tachyphylaxis to intravenous isoprenaline by measuring both heart rate and bronchodilator effects in the same animal. Bronchodilator effects were also measured during repeated doses of an isoprenaline aerosol. A preliminary report of this work has been presented (Minatoya & Spilker, 1973).

Methods

A total of 81 mongrel dogs (9 to 13 kg) of either sex was anaesthetized with pentobarbitone Na (30 mg/kg, i.v.) followed 30 min later by morphine sulphate (3 mg/kg, i.m.). The open-chest dog was maintained by artificial respiration (14 to 22 strokes/min of 250 ml each). A specially designed non-rebreathing plastic valve was modified from one previously reported (Minatoya & Luduena, 1967) and was attached to a tracheal cannula so that expired air returned to the atmosphere. Previously conducted experiments using the non-rebreathing valve, demonstrated that open-chest dogs under artificial respiration gave more consistent recordings of intratracheal pressure, bronchoconstriction and bronchodilation than did closed-chest dogs. CO_2 in expired air was continuously monitored in previous as well as present experiments by the Beckman model LB-1 CO_2 gas analyser and was in the range of 4.0 to 5.0%. A volume of 250 ml was used to respire dogs since this volume maintained blood pressure, heart rate and CO_2 in expired air at a nearly constant level throughout a 6 h experiment in many other dogs. A Statham P23B pressure transducer was attached to the side arm of the tracheal cannula and the intra-tracheal pressure recorded on a Grass Model 7 polygraph. Aerosols were delivered directly into the trachea during inspiration through an actuator attached to the cannula. Heart rate was monitored with a Sanborn model 300 portable ECG apparatus using lead II. Blood pressure was measured from the right femoral artery by a Statham P23A pressure transducer and recorded on the polygraph. The left femoral vein was cannulated for infusions and the right femoral vein cannulated for injections.

Bronchoconstriction was induced with histamine diphosphate. Doses ranged from 25 to 50 µg/kg i.v., but were kept constant during each experiment. The degree of bronchoconstriction was determined by measuring the area of the intratracheal pressure recordings above the baseline for a 5 min period after the histamine injection. Paper speed was 10 mm/min and a planimeter was used to measure this area. Bronchodilation was expressed as percentage inhibition of the two control histamine-induced bronchoconstrictions.

Drugs used were (±)-isoprenaline hydrochloride (Sterling-Winthrop) and histamine diphosphate (Schwarz-Mann). All doses are in terms of base. Drug solutions for injection were prepared daily in distilled water and kept on ice. Isoprenaline solutions for infusion were prepared in 0.9% w/v NaCl (saline) and infused at a rate of 0.1 ml/min using a Harvard model 600 constant infusion apparatus with a 10 ml syringe. Pressurized aerosol solutions of isoprenaline, delivering 63 or 250 µg per actuation, were used

Analysis of variance determinations were performed on a PDP-15 computer (Digital Equipment).

Results

Heart rate responses to isoprenaline before and after a 15 min infusion of isoprenaline

The heart rate response to injections of different doses of isoprenaline was determined before and after its infusion for 15 minutes. Three injection doses of isoprenaline (0.0125, 0.05 and 0.2 µg/kg) were tested and three different infusions were given (0.007 µg kg^{-1} min^{-1}, $n = 5$; 0.021 µg kg^{-1} min^{-1}, $n = 6$; and 0.063 µg kg^{-1} min^{-1}, $n = 6$). Sufficient time was allowed between each of the three injections (10 to 15 min) for the heart rate to approximate control. After a 15 min infusion, the heart rate was allowed to return to within 10 beats of the control value just prior to infusion before the second dose-response was begun (usually 10 to 15 minutes). Each dose-response procedure took 30 to 40 min to perform. Heart rate increases to isoprenaline obtained before and after each isoprenaline infusion were essentially unchanged (Table 1).

Bronchodilation and heart rate responses to isoprenaline before and after a 30 min infusion of isoprenaline

Heart rate and bronchodilator responses to injections of isoprenaline (1 µg/kg, i.v.) obtained

Table 1 Heart rate responses to isoprenaline (i.v.) before and after a 15 min infusion of isoprenaline.

Infusion rate (µg kg^{-1} min^{-1})	*Pre-infusion increase in heart rate (beats/min)*			*Post-infusion increase in heart rate (beats/min)*		
	0.0125 µg/kg	*0.05 µg/kg*	*0.2 µg/kg*	*0.0125 µg/kg*	*0.05 µg/kg*	*0.2 µg/kg*
0.007	13 ± 2 (100)	42 ± 6 (104)	92 ± 17 (104)	16 ± 3 (113)	42 ± 9 (113)	90 ± 17 (111)
0.021	20 ± 3 (117)	48 ± 5 (121)	101 ± 10 (123)	24 ± 2 (128)	56 ± 6 (127)	94 ± 5 (129)
0.063	16 ± 3 (118)	59 ± 9 (112)	86 ± 13 (112)	17 ± 3 (128)	48 ± 10 (126)	77 ± 15 (125)

The numbers in parentheses are the heart rates just prior to the isoprenaline injection. Each value ± s.e.m. is derived from 5 or 6 experiments. The post-infusion increase in control heart rate was not significant when analysed by a paired *t* test.

in the same animal before and after isoprenaline infusions for 30 min were determined (Table 2). Two doses of isoprenaline were infused (0.007 $\mu g\ kg^{-1}\ min^{-1}$, $n = 6$; and 0.021 $\mu g\ kg^{-1}\ min^{-1}$, $n = 6$). Histamine was injected 45 s after the isoprenaline injection to allow time for the maximum increase in heart rate to be observed. Linear contrasts within a row by column analysis of variance demonstrated that there were no significant differences ($P > 0.05$) between the 10 and 30 min values for heart rate. Also, there were no significant differences between these values and the mean value of control heart rate ($P > 0.05$). Similarly, there were no significant differences in the degree of bronchodilation before and after the infusion of isoprenaline.

The control heart rates of dogs used in this experiment were higher than those used in experiments reported in Table 1. This may be due to the fact that the two experiments were conducted at different times of the year with dogs of different breeds.

Heart rate responses during a 4 h infusion of isoprenaline with a concomitant injection of isoprenaline every 30 minutes

Heart rate responses during a 4 h infusion of isoprenaline and to concomitant injections of isoprenaline (0.05 μg/kg i.v.) every 30 min during the infusion are shown in Figure 1. Three infusion doses were given ($n = 4$ per dose) and there were dose-related increases in heart rate which reached a plateau in approximately 15 minutes. The elevated heart rates remained relatively constant throughout the infusion period. Heart rate increases to single injections of isoprenaline given on top of the infusion also remained relatively constant and the amplitude was dependent upon the heart rate level (Figure 1). An infusion of 0.175 $\mu g\ kg^{-1}\ min^{-1}$ caused the heart rate to plateau at approximately 220 beats/minute. At this heart rate, an increase of only 5 to 10 beats/min was obtained to isoprenaline injections, whereas an increase of 38 to 47 beats/min was obtained with the lowest infusion dose (0.007 $\mu g\ kg^{-1}\ min^{-1}$) where the heart rate was about 120 beats/minute.

Bronchodilation and heart rate responses during a 5 h infusion of isoprenaline

Heart rate and bronchodilator effects were studied concurrently in the same animal during a 5 h infusion. Responses to histamine were determined before, 5 min after and at hourly intervals during the infusion of isoprenaline. Heart rates were monitored 5 to 10 min prior to each injection of histamine. No decrease of the bronchodilator

Table 2 Heart rate and bronchodilator responses to isoprenaline (1 μg/kg, i.v.) before and after a 30 min infusion of isoprenaline.

	Heart Rate				*Bronchodilation*			
Infusion rate ($\mu g\ kg^{-1}\ min^{-1}$)	*Pre-infusion (beats/min)*	*10 min. Post-infusion (beats/min)*	*30 min. Post-infusion (beats/min)*	*s.e.m.*	*Pre-infusion (%)*	*10 min. Post-infusion (%)*	*30 min. Post-infusion (%)*	*s.e.m.*
0.007	73 (154 ± 7)	68 (157 ± 7)	65 (160 ± 11)	±5.0	51	43	46	±2.5
0.021	88 (151 ± 13)	78 (155 ± 13)	76 (159 ± 10)	±4.3	53	44	40	±10.3

The numbers in parentheses are the heart rates ± s.e.m. ($n = 6$) just prior to the isoprenaline injection. The s.e.m. values for heart rate increases and % bronchodilation are based on pooled variance estimates from analysis of variance.

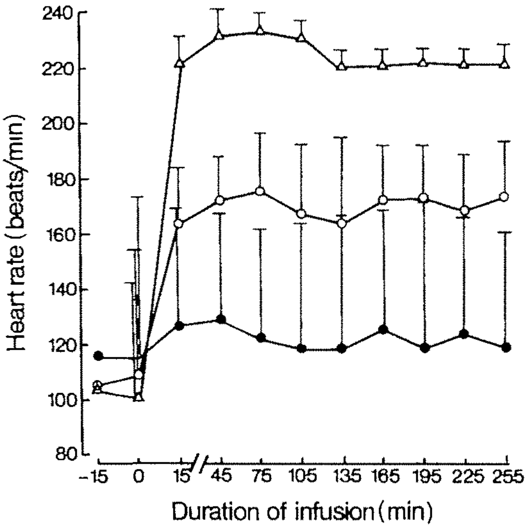

Figure 1 Heart rate responses to infusions of isoprenaline and to single injections of isoprenaline (0.05 μg/kg) every 30 min during the infusion. The effect of each injection of isoprenaline is shown as an upward line. (△) Isoprenaline (0.175 μg kg^{-1} min^{-1}, $n = 4$); (○) Isoprenaline 0.035 μg kg^{-1} min^{-1}, $n = 4$); (●) Isoprenaline (0.007 μg kg^{-1} min^{-1}, $n = 4$).

response to isoprenaline was observed (Figure 2). A consistent bronchodilation of about 75% was maintained for 5 h with the larger infusion dose (0.175 μg kg^{-1} min^{-1}, $n = 7$) and a bronchodilation of 15-30% with the smaller dose (0.007 μg kg^{-1} min^{-1}, $n = 4$). The elevated heart rate remained constant throughout the infusion period. Substituting physiological saline for histamine during the isoprenaline infusion did not alter the heart rate effect (Figure 2, $n = 4$).

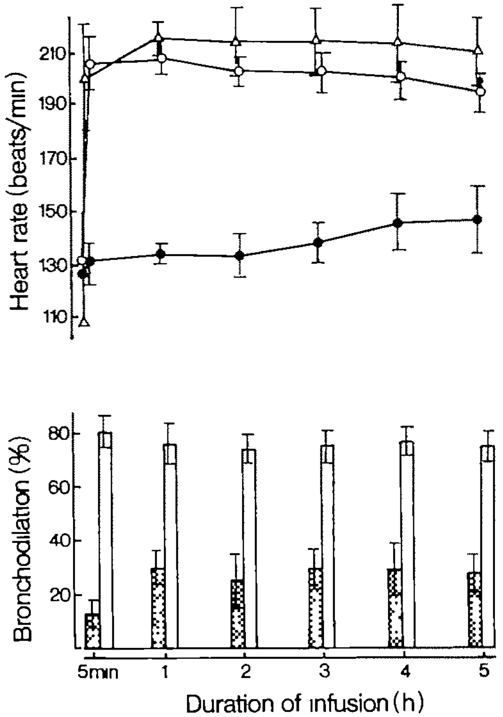

Figure 2 The upper trace shows heart rate increases ± s.e.m. to infusions of isoprenaline where injections of histamine or saline were made each hour. (△) Isoprenaline (0.175 μg kg^{-1} min^{-1}) with saline injections ($n = 4$); (○) Isoprenaline (0.175 μg kg^{-1} min^{-1}) with histamine injections ($n = 4$); (●) Isoprenaline (0.007 μg kg^{-1} min^{-1}) with histamine injections ($n = 4$). The lower trace shows bronchodilator effects (% antagonism of the histamine-induced bronchoconstriction) in the same animals. Open columns, isoprenaline (0.175 μg kg^{-1} min^{-1}); stippled columns, isoprenaline (0.007 μg kg^{-1} min^{-1}).

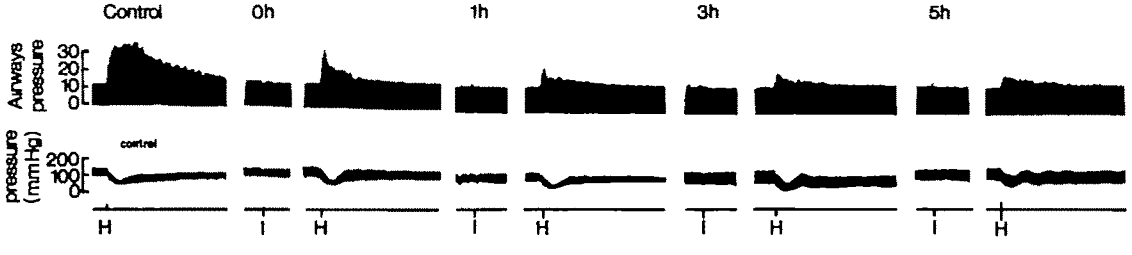

Figure 3 Bronchodilator effects of repeated intratracheal administrations of isoprenaline aerosol in an 11.2 kg female dog. A dose of 500 μg (2 actuations) was given each hour (I). Airways pressure is recorded as mm from polygraph trace Histamine (45 μg/kg i.v.) was tested hourly 15 min after each aerosol medication (H). Respiration frequency was 15/minute.

Table 3 Bronchodilator effects of repeated intratracheal administrations of isoprenaline aerosol.

Isoprenaline Dose (µg)	*Frequency (min)*	*Bronchodilation (%) at various times (min) after the first dose* 2	*15*	*45*	*62*	*75*	*80*	*105*	*122*	*135*	*165*	*180*	*182*	*195*	*225*	*242*	*255*	*280*	*302*	*315*	*320*
250	Every 30		30	25		23		42		23	36			46	44						
500	Every 30		46	48		54		56		61	52			57	60		57				
1000	Every 30		47	51		53		52		59	58			48	54		62				
250	Every 60		12			29				28				28			26				
500	Every 60		58			69				52				68			58			61	
1000	Every 60		52			47				50				60			67			66	
2000	Every 60		72			74				71				72			71				
1000	Every 60	82			85				82				88			87			85		
2000	Every 60	97			95				95				88			90			89		
250	Every 20						30					32						33			32
63	Every 20						17					20						19		14	

Each value of bronchodilation represents percent inhibition of the histamine (i.v.) challenge.
Histamine injections were given at 2, 15, or 20 min after each medication.
The actuator delivered 250 µg/actuation except in the last experiment where it was 63 µg/actuation.
Each experiment was performed in 2-4 dogs (total = 25 dogs) and results were averaged.

Bronchodilation studies during repeated aerosol medications of isoprenaline

Figure 3 shows a polygraph tracing obtained in a dog (11.2 kg) receiving 500 µg of isoprenaline aerosol (2 actuations) each hour for 5 hours The histamine challenge was given 15 min after each aerosol medication. At the initial measurement (zero hour) there was a 68% bronchodilation. Thereafter, consistent bronchodilations of about 75% were obtained.

Table 3 summarizes the aerosol results. Doses ranging from 63 to 2000 µg (1-8 actuations) were administered every 20 to 60 min for 4-5 hours. The intravenous histamine challenge was given 2, 15 or 20 min after each aerosol administration and the results were expressed as percentage inhibition (bronchodilation) of the control histamine response. No evidence of bronchodilator tachyphylaxis was observed ($n = 25$). There were no differences in the results irrespective of whether histamine was injected 2 or 15 min after each aerosol actuation(s) except that the degree of bronchodilation was greater when tested at 2 minutes

Discussion

The present results showed no evidence of bronchodilator or heart rate tachyphylaxis to isoprenaline in healthy dogs using various experimental procedures. Conolly *et al.* (1971) reported that tachyphylaxis occurred to isoprenaline in dogs. This discrepancy could be due to differences in experimental conditions. For example, Conolly *et al.* (1971) used closed-chest dogs not under artificial respiration. The average dose of isoprenaline required to increase heart rate by 40 beats/min in control dogs was greater than 1.0 µg/kg in their experiments, but less than 0.05 µg/kg in the present experiments. Other reported values for this effect are about 0.1 µg/kg (Kofi Ekue, Shanks & Zaidi, 1971) and 0.7 µg/kg (Daly, Farmer & Levy, 1971). These doses are in terms of base.

There is considerable controversy concerning the cause of the increased death rate in asthmatic patients experienced in certain countries during the 1960's (Harris, 1971; Stolley, 1972; Silverglade, 1972; Herxheimer, 1972b; Gandevia, 1973; Spilker, 1973). Conolly *et al.* (1971) suggested that tachyphylaxis to the β-adrenoceptor effects of nebulized bronchodilators and a drug-induced cross tachyphylaxis to endogenous sympathetic stimulation could have led to deterioration of the asthmatic patient. One of us has previously questioned this hypothesis (Spilker, 1973).

Conolly *et al.* (1971) were unable to explain their finding that an infused dose of only 0.01 to 0.86 μg/kg isoprenaline caused a significant shift of the isoprenaline dose-response curve to the right in 9 of 11 dogs and caused a nearly 3-fold increase in the dose of isoprenaline required to increase the heart rate by 40 beats/minute. In humans, an infused dose of 0 055 to 0 45 μg/kg isoprenaline also caused a significant shift of the dose-response curve in 6 of 7 subjects and more than doubled the dose required to increase heart rate by 20 beats/minute The magnitude of these effects was *not* related to the size of the infused dose. The infused dose of isoprenaline in our study varied from 0.105 to 52.50 μg/kg, but in no case was tachyphylaxis observed, either during the infusion or to a dose-response relationship for isoprenaline determined after the infusion

Paterson, Conolly, Davies & Dollery (1968) reported that normal subjects experienced tachycardia to therapeutic doses of isoprenaline aerosol whereas asthmatic patients did not. However, it has often been shown that heart rate increases to isoprenaline aerosol are minimal or absent in asthmatics while bronchodilator responses are marked (Hambleton & Shinebourne, 1970; Streeton, 1970, Riding, Chatterjee, Bernstein & Dinda, 1971; Freedman & Hill, 1971). Further, Paterson *et al.* (1968) did not examine bronchodilator responses in their group of asthmatic patients. Thus, even if tachyphylaxis to heart rate effects of isoprenaline were valid, it could not be used to support the view that bronchodilator tachyphylaxis was also present and then to suggest that the observed rise in asthma deaths in England and Wales could be explained, as did Conolly *et al.* (1971), by tachyphylaxis to isoprenaline. Morton & Ostensoe (1963) and Pierson & Grieco (1969) reported that heart rate responses of normals and asthmatics to inhaled isoprenaline were equal. Cookson & Reed (1963) reported that there was no significant difference between heart rate responses of normals and asthmatics to three doses of infused (i.v.) isoprenaline. Kingsley *et al.* (1972) were unable to demonstrate tachyphylaxis to the acute heart rate effects of isoprenaline in normal human subjects.

Atkinson & Rand (1968) observed decreased heart rate effects to injected isoprenaline (as well as to other catecholamines) given during an isoprenaline infusion in cats (Figure 5 of their paper). The heart rate response to injected isoprenaline was also decreased when it was injected during orciprenaline infusion (Figure 6 of their paper). These reduced responses to isoprenaline injections would be expected on the basis of the increased background heart rates observed during the infusion and are not an example of tachyphylaxis. The reduced heart rate response to injected isoprenaline given while the heart rate was elevated is also seen in Figure 1 of this paper. A similar observation has been made in man (Kingsley *et al.*, 1972).

The possibility of isoprenaline-induced bronchodilator tachyphylaxis has been previously studied. Conolly *et al.* (1971) reported that guinea-pigs developed tachyphylaxis to isoprenaline after repeated injections over 5 hours. Tachyphylaxis was shown by the increased mortality of isoprenaline treated guinea-pigs to histamine challenge. This study was confirmed by Bouhuys *et al.* (1972). However, the increased mortality was statistically significant in only one of three groups of guinea-pigs treated with isoprenaline in both the study of Conolly *et al.* (1971) and that of Bouhuys *et al.* (1972). The degree of mortality after histamine may have been influenced by haemodynamic changes caused by the doses of isoprenaline used (4 μg/kg i m.—every 20 min for 5 h in the study of Bouhuys *et al.* and every 20 to 45 min for 5 h in the study of Conolly *et al.*). Thus the relevance of these results to isoprenaline tachyphylaxis will be better understood when haemodynamic data are available. No evidence of tachyphylaxis to bronchodilator responses was observed upon repeated isoprenaline injections during a 2.5 h isoprenaline infusion in guinea-pigs (McCulloch, Pun & Rand, 1968: Pun *et al.*, 1971). Repeated applications of isoprenaline in concentrations greater than 5×10^{-5} M did *not* consistently produce tachyphylaxis in more than 50 tracheal preparations from both young and old rats (Fleisch & Titus, 1972). Sobol, Emirgil, Wadhwani & Goyal (1972) followed 32 patients with chronic obstructive pulmonary disease for 4 to 32 months (average 18 months) who used an isoprenaline nebulizer at least 5 times/day and found that bronchodilator effects did not diminish. Ayres (1973) studied 350 patients over a 4 year period and did not observe any evidence of tachyphylaxis to nebulized isoprenaline.

There are several reports of patients in status asthmaticus who do not respond favourably to sympathomimetic bronchodilators (Keighley, 1966, Reisman *et al.*, 1968; Van Metre, 1969). There are also some patients whose condition may deteriorate when such aerosols are used (Keighley, 1966). It is important to determine whether patients who do not respond to bronchodilator aerosols are tolerant to their effects because of over-use or for other reasons. Although this question cannot be definitively answered at present, there is some evidence that asthmatics are not tolerant solely because of aerosol over-use. An editorial in 'The British Medical Journal' (1972) points out that in cases of status asthmaticus

'Bronchodilator aerosols have ceased to be effective' and 'Sympathomimetic bronchodilators, by inhalation or by injection, should be avoided unless oxygen can be administered at the same time.' Possible reasons to explain the lack of effect obtained with bronchodilators in severe asthma include impaired ventilation/perfusion ratios due to abnormal blood gas tensions which isoprenaline does not always correct (Palmer & Diament, 1967; Waddell, Emerson & Gunstone, 1967) and severe mucous plugs in the bronchi which are unaffected by isoprenaline (Harris, 1972). Severely asthmatic patients thus may reach a bronchodilator tolerant stage, independent of any prior use of aerosols

Our conclusion, therefore, is that neither heart rate nor bronchodilator tachyphylaxis to isoprenaline has been unequivocally demonstrated in animals or man.

Note added in proof

After this paper went to press, another paper appeared (McDevitt, Shanks & Swanton, *Br J. Pharmac* (1974), **50**, 335-344) in which no evidence of significant tachyphylaxis to isoprenaline in dogs was observed

Aerosol preparations were kindly supplied by Dr William Gorman and his staff These experiments were carried out with the able assistance of Miss Mary Gosztyla, Dr Eugenio Bogado and Mrs Joyce Tyll.

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AN INVESTIGATION OF THE TACHYCARDIA PRODUCED BY INTRACEREBROVENTRICULAR INJECTIONS OF ISOPRENALINE IN MICE

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1 Isoprenaline, 3.5-20 ng, injected intracerebroventricularly in atropinized mice under pentobarbitone anaesthesia produced a dose-dependent tachycardia.

2 Pretreatment with either reserpine or pempidine blocked nervously-mediated tachycardia as shown by marked reduction of that due to stimulation of the spinal outflow in pithed mice. After pretreatment with these drugs, intracerebroventricular isoprenaline caused tachycardia of a similar degree and time course to that in mice not so pretreated.

3 Pretreatment with either reserpine or pempidine caused supersensitivity to the tachycardia due to intravenous isoprenaline.

4 When allowance was made for this supersensitivity in the effect of intracerebroventricular isoprenaline in pretreated mice, a small dose-dependent residual effect remained that could be attributed to leakage of isoprenaline into the peripheral circulation.

5 This was confirmed by the appearance of a late-developing tachycardia on intracerebroventricular injection of isoprenaline in spinal mice.

6 It is therefore concluded that the tachycardia caused by intracerebroventricular isoprenaline in mice is, at least initially, of central origin.

Introduction

Evidence suggesting the existence of central adrenoceptors has been available since 1960, when Page and his co-workers (McCubbin, Kaneko & Page, 1960; Kaneko, McCubbin & Page, 1960) showed that intracerebroventricular noradrenaline caused cardiovascular effects susceptible to α-adrenoceptor blockade, though these authors suggested that these were indirectly due to vascular changes. Share & Melville (1964) demonstrated that injection of picrotoxin into the lateral cerebral ventricle of the cat induces sympathetic stimulation, producing cardiovascular changes mediated via a release of brain stem noradrenaline. In later experiments (Share & Melville, 1965), it was shown that while the hypotension observed after picrotoxin injection could be abolished by intracerebroventricular phenoxybenzamine, the tachycardia remained unaffected. Intracerebroventricular dichloro-isoprenaline reduced the tachycardia, while the hypotension remained unchanged.

Intracerebroventricular injection of isoprenaline in anaesthetized cats has been shown to produce tachycardia and hypotension, these effects being abolished by pretreatment with pronethanol or propranolol by the same route (Gagnon & Melville, 1967). From this evidence it was suggested that sympathetic receptors exist in central regions paralleling those postulated in the periphery by Ahlquist (1948).

Bhargava, Mishra & Tangri (1972) have shown these effects of intracerebroventricular isoprenaline to be central in origin in anaesthetized dogs by experiments in which bilateral removal of the stellate ganglion or transection of the upper spinal cord was carried out. Using conscious cats Day & Roach (1972) showed that intracerebroventricular isoprenaline produced tachycardia and hypertension, the latter being due to an increased systolic pressure. These effects could be blocked by peripheral injection of pempidine or intracerebroventricular injection of propranolol.

Intracerebroventricular injection of agents in mice (Haley & McCormick, 1957; Brittain, 1966) can be followed by leakage into the general circulation to an extent greater than that indicated by the injection of opaque materials (Shaw, 1974), leading to otherwise unsuspected systemic effects. Cowell & Davey (1968) have shown that the effects of intracerebroventricularly injected noradrenaline are mediated partly by a fraction that leaks to the periphery.

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In the present studies, we have investigated the possibility that the effects of intracerebroventricularly injected isoprenaline in the mouse may be produced in this way, rather than by direct interaction with central β-adrenoceptors.

Method

Male CFW mice were anaesthetized with sodium pentobarbitone, 100 mg/kg intraperitoneally, 15 min after pretreatment with atropine methylbromide, 2 mg/kg subcutaneously. Each animal was placed on a warming pad and electrodes were inserted in the skin of the chest wall to record the ECG. From this the heart rate was measured using an ECG preamplifier and a Devices ratemeter.

Fifteen min after the pentobarbitone, the control heart rate was read for each mouse and isoprenaline in a volume of 10 μl of solution was injected intracerebroventricularly, in a manner similar to that described by Haley & McCormick (1957). A 0.5 ml Hamilton syringe with a No. 20 needle, 3 mm long was used. The needle was inserted vertically through a point in the skull on the parieto-frontal suture. Examination after the injection of ink showed material in the lateral and third ventricles.

Control animals were injected with 0.9% w/v NaCl solution (saline) intracerebroventricularly and were included in each experiment. All solutions injected intracerebroventricularly were warmed to 37°C before administration. Readings of heart rate were taken at 1, 3, 5, 7, 10, 12 and 15 min after injection.

Some animals were pretreated with reserpine, 3 mg/kg intraperitoneally, 18 h before use, while others received pempidine, 5 mg/kg intraperitoneally, 30 min before use.

In some experiments, mice were pithed and the spinal outflow stimulated electrically by the method of Gillespie & Muir (1967). These animals were pithed with a blunted serum needle inserted through the orbit after cannulation of the trachea. This was carried out under ether anaesthesia after pretreatment with atropine methylbromide.

The tracheal cannula was then connected to a Palmer respiration pump and the animal respired at 200 strokes/min at a pressure of 10 cm water. The animals were then bilaterally adrenalectomized. The spinal outflow was stimulated electrically, through a needle placed in a hind limb to act as an indifferent electrode and with a Grass stimulator connected to the pithing rod to deliver, for 15 s, a square wave stimulation of 10 Hz, 2 ms pulse width and variable voltage; pretreatment with (+)-tubocurarine, 2 mg/kg intraperitoneally, was given to reduce skeletal muscle twitches.

In later experiments, spinal mouse preparations were used. The animals were again pretreated with atropine methylbromide, (2 mg/kg s.c.), and given pentobarbitone, (100 mg/kg i.p.). Ten min later, a tracheal cannula was inserted and a loop of nylon line drawn round the spinal column, ensuring that the carotid arteries were not included. By tightening the loop, severance of the spinal cord occurred, as judged by the cessation of respiration. These animals were then artificially respired as described earlier. Each animal was allowed 15 min to equilibrate after spinalization and either isoprenaline or saline was then injected intracerebroventricularly, the heart rate being measured as previously described. For these experiments, intact control animals were used, pretreated with atropine methylbromide and pentobarbitone and left for 30 min before being injected with saline or isoprenaline intracerebroventricularly.

Drugs used were. atropine methylbromide (IC Pharmaceuticals), pentobarbitone sodium (Abbott), isoprenaline sulphate B.P. (Burroughs Wellcome), (+)-tubocurarine chloride (Burroughs Wellcome), reserpine (Ciba), pempidine tartrate (May & Baker).

Results

Effect of intracerebroventricular isoprenaline

After pretreatment with atropine methylbromide, the effect of pentobarbitone anaesthesia was to produce a gradual decline in the resting heart rates of mice over 25 min (Figure 1). After injecting saline intracerebroventricularly there was a further, more abrupt fall in heart rate which lasted for 6-7 min (Figure 1). This phenomenon was also observed after intracerebroventricular introduction of a 'dry needle'; thus the abrupt fall in heart rate observed with saline was a consequence of the injection procedure.

The effect of an intracerebroventricular injection of isoprenaline was a dose-dependent tachycardia followed by a gradual decline, despite the bradycardia produced by the act of injection. The net effects of the intracerebroventricular isoprenaline were calculated as the difference from control animals similarly injected with saline (ΔHR, Figure 2).

Effect of pretreatment with agents blocking the sympathetic nervous system

In order to determine the peripheral contribution to tachycardia from any amine leaking into the

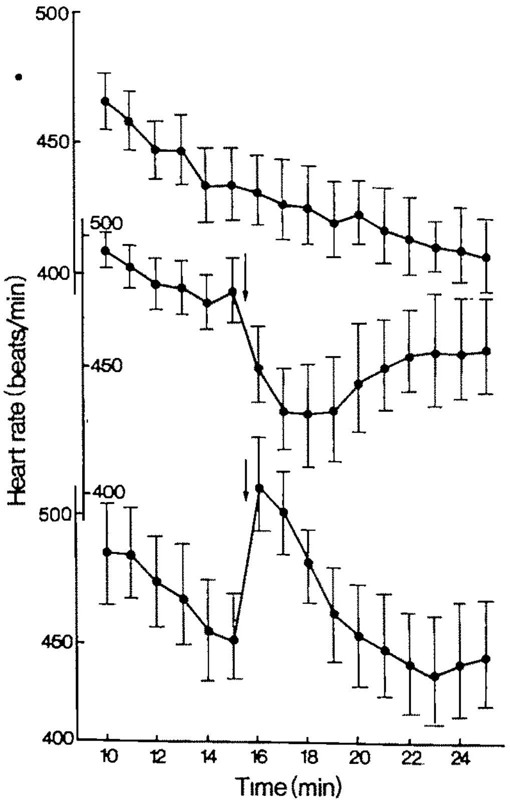

Figure 1 Mean heart rate of groups of mice, read at 1 min intervals from 10 min after injection with pentobarbitone sodium, (100 mg/kg i.p.); 15 min before this they had received atropine methylbromide (2 mg/kg s.c.). From above downwards: no further treatment; intracerebroventricular injection of saline, 10 μl, at arrow; intracerebroventricular injection of isoprenaline, 10 ng in 10 μl, at arrow. Vertical lines indicate s.e. mean.

general circulation, mice were pretreated with reserpine, (3 mg/kg i.p.) 18 h before the intracerebroventricular isoprenaline administration, to block the central component of the effect. This reduced the resting heart rate from 414.3 ± 8.3 to 243.8 ± 11.2 beats per minute.

After this pretreatment, intracerebroventricular isoprenaline produced a dose-dependent tachycardia very similar to that observed in non-reserpine-treated animals (Figure 3). These results can usefully be compared by plotting against dose the sum of ΔHR for all readings taken over 15 min after injection. ($\Sigma\Delta HR = \Delta HR_1 + \Delta HR_3 + \Delta HR_5 + \Delta HR_7 + \Delta HR_{10} + \Delta HR_{12} + \Delta HR_{15}$). Figure 4 suggests that reserpine pretreatment increased rather than reduced the degree of tachycardia due to intracerebroventricular isoprenaline administration.

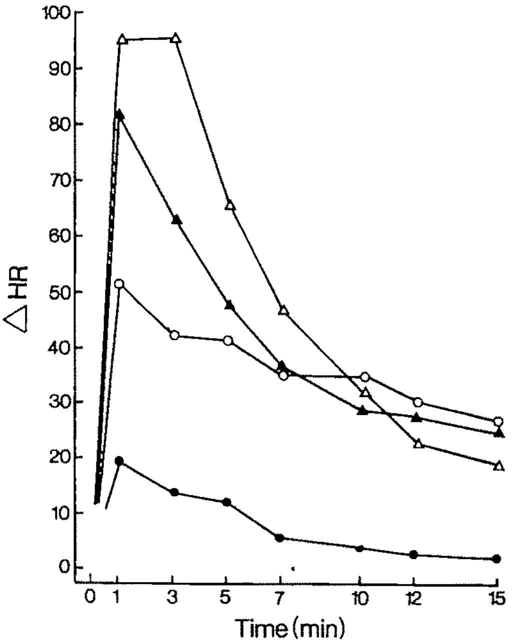

Figure 2 The effect upon the mean heart rate of groups of mice of various doses of isoprenaline injected intracerebroventricularly after pretreatment with atropine methylbromide and anaesthesia with pentobarbitone sodium, as in Figure 1. ΔHR = mean of difference between individual values and mean for control group, injected intracerebroventricularly with saline, read at the same time. Isoprenaline (●) 3.5 ng ($n = 49$); (○) 5 ng ($n = 49$); (▲) 10 ng ($n = 50$); (△) 20 ng ($n = 25$).

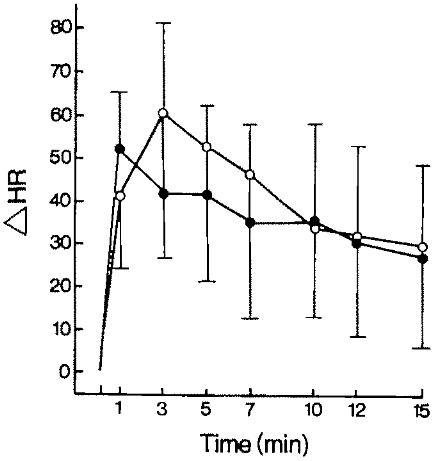

Figure 3 The effect upon heart rate of 5 ng isoprenaline, injected intracerebroventricularly in groups of mice anaesthetized with pentobarbitone sodium and pretreated with atropine methylbromide, with (○) or without (●) additional pretreatment with reserpine (3 mg/kg i.p.) 18 h earlier.

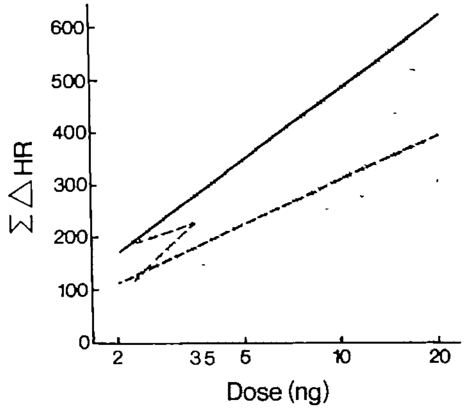

Figure 4 Dose-response relations for the effect upon heart rate of intracerebroventricularly injected isoprenaline in groups of mice anaesthetized with pentobarbitone sodium and pretreated with atropine methylbromide, with (unbroken line) or without (broken line) additional pretreatment with reserpine, as in Figure 3.

$\Sigma\Delta\ HR = \Delta\ HR_1 + \Delta\ HR_3 + \Delta\ HR_5 + \Delta\ HR_7 + \Delta\ HR_{10} + \Delta\ HR_{12} + \Delta\ HR_{15}$, where subscripts denote minutes after i.c.v. injection.

The shaded areas denote the 95% confidence limits. For reserpine-treated mice $n = 119$; for mice not treated with reserpine $n = 198$.

Isoprenaline injected intravenously into reserpine-pretreated mice was found to produce an even greater tachycardia than in non-reserpine-treated animals (Figure 5). This may be seen by the displaced relation between dose and response in terms of ΣΔ HR when the increase in heart rate due to intravenous isoprenaline in normally sensitive mice was plotted against that in mice rendered supersensitive by pretreatment with reserpine (Figure 6). It appeared that levels of circulating isoprenaline that normally cause a measurable tachycardia caused a greater tachycardia in reserpine-treated mice by a fixed amount (ΔHR = 63.5 beats/min) at all time intervals; normally sub-threshold levels of circulating isoprenaline cause tachycardia of less than ΔHR = 63.5 beats/min in reserpine-treated mice.

As an alternative means of blocking the central components, mice were pretreated with pempidine, (5 mg/kg i.p.) 30 min before injection of isoprenaline, which reduced the resting heart rate from 418.1 ± 4 to 303.4 ± 8.9 beats/minute. Here, no apparent difference between the pempidine-treated and control animals was observed but, once again, a much greater sensitivity to intravenous isoprenaline was found in the pretreated animals, although the maximum tachycardia that could be produced after pretreatment was reduced.

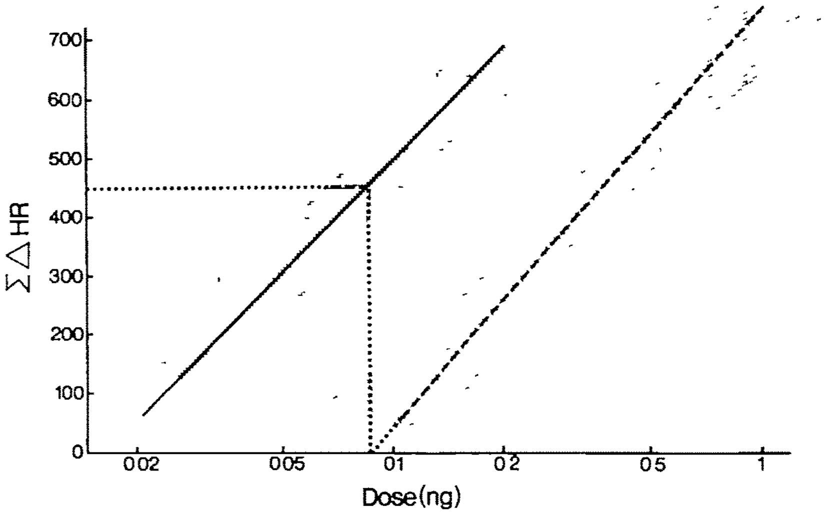

Figure 5 Dose-response relations for the effect upon heart rate of intravenously injected isoprenaline in reserpine-treated (unbroken line) and non-reserpine-treated mice (broken line), anaesthetized with pentobarbitone sodium and pretreated with atropine methylbromide. Each isoprenaline injection took approximately 2 s and the responses were measured over the subsequent 15 minutes. The shaded areas denote the 95% confidence limits. For reserpine-treated mice $n = 53$; for mice not treated with reserpine $n = 50$.

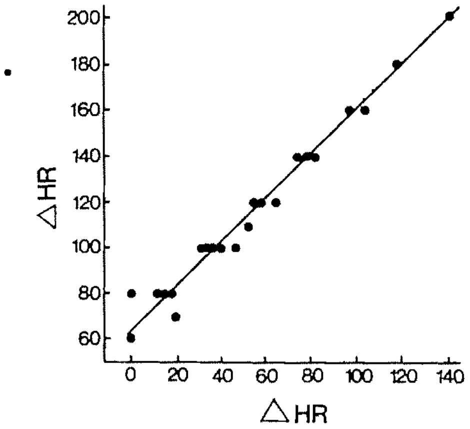

Figure 6 The relation between Δ HR values in groups of mice, measured at various intervals up to 15 min after the intravenous injection of isoprenaline following anaesthesia with pentobarbitone sodium and pretreatment with atropine methylbromide (abscissae), and corresponding values in mice additionally pretreated with reserpine, 3 mg/kg, 18 h earlier (ordinates). ($r = 0.986$, $n = 22$ pairs; $P < 0.001$).

Effect of pithing and adrenalectomy

The effectiveness of the doses of reserpine and pempidine in blocking the sympathetic nervous system was checked by using adrenalectomized mice and electrically stimulating the thoracic spinal outflow via the pithing rod. These experiments were carried out in atropinized mice, with and without pretreatment by reserpine or pempidine, and the maximum increase in heart rate produced by electrical stimulation for each animal was plotted against the voltage required to produce the effect. After pretreatment, an increase in heart rate beyond 30 beats/min could not be produced by stimulation with up to 100 V, whereas the minimum increase in control mice was 50 beats/minute.

Effect in spinal mice

Mice were spinalized as a further alternative means of blocking tachycardia of central origin. This did not affect the resting heart rate (from 397 ± 29 to 407 ±7.3 beats/minute). On injection of isoprenaline intracerebroventricularly, tachycardia developed only towards the end of the 15 min of observation (Figure 7). However, the response to intravenous isoprenaline in such mice was significantly greater than that in untreated mice (Figure 8), though less so than after reserpine- or pempidine-pretreatment.

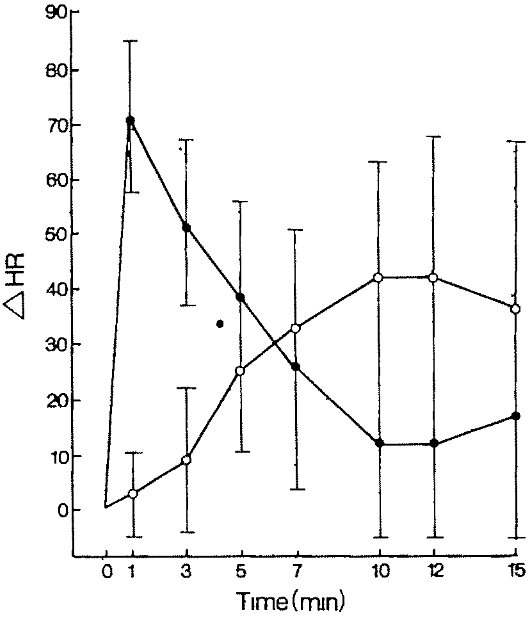

Figure 7 The effect upon heart rate of 10 ng isoprenaline injected intracerebroventricularly in groups of spinal (○) or intact mice (●), anaesthetized with pentobarbitone sodium and pretreated with atropine methylbromide. The vertical lines indicate s.e. mean.

Discussion

The poor responsiveness of mice pretreated with reserpine or pempidine to electrical stimulation of the thoracic cord outflow confirms that this pretreatment was adequate to block nervously-mediated tachycardia. The effect produced by intracerebroventricular isoprenaline in such mice must therefore be attributed to a direct action on the heart of amine leaking into the circulation.

The similarity between the effect of intracerebroventricular isoprenaline in untreated mice and that in mice pretreated with blocking agents suggests at first that this, too, is an effect consequent upon leakage. Two findings argue against this, however. It has been consistently observed that the increase in heart rate in reserpine-treated mice reaches a peak level later than in untreated mice. The values in untreated mice decline from 1 min after injection (Figure 3), suggesting that the two effects are not entirely identical.

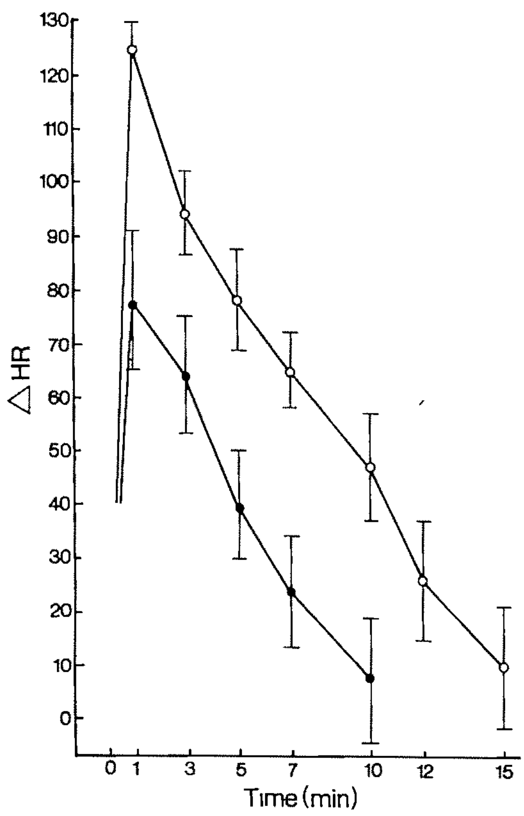

Figure 8 The effect upon heart rate of 0.5 ng isoprenaline injected intravenously in groups of spinal (o) or intact mice (•), anaesthetized with pentobarbitone sodium and pretreated with atropine methylbromide. The vertical lines indicate s.e. mean.

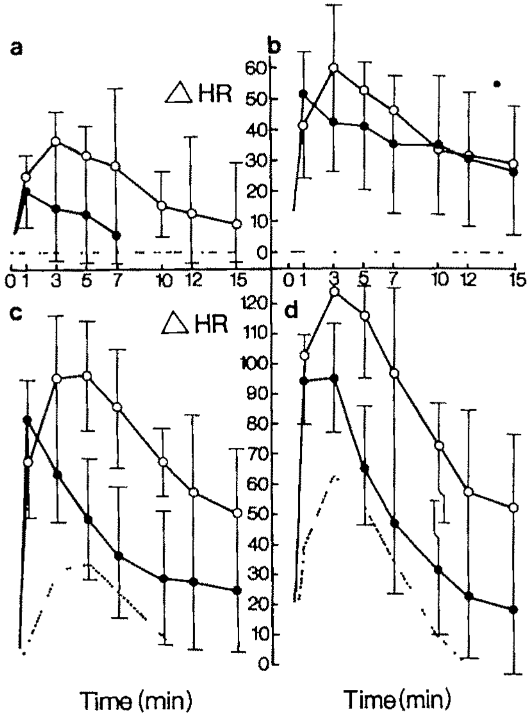

Figure 9 The effect upon the mean heart rate of groups of mice of doses of isoprenaline injected intracerebroventricularly after pretreatment with atropine methylbromide and anaesthesia with pentobarbitone sodium, with (o) or without (•) additional pretreatment with reserpine. The dotted line indicates the residual effect in reserpine-treated mice after correction for supersensitivity. Vertical lines indicate s.e. mean. Isoprenaline (a) 3.5 ng, (b) 5 ng, (c) 10 ng, (d) 20 ng. Section (a) is identical with Figure 3.

It has also been shown that pretreatment with reserpine results in supersensitivity to intravenous isoprenaline, such that the amine now appears five times as potent in causing tachycardia (Figure 5). A similar increase in response to isoprenaline was reported by Barrett & Carter (1970) in rats pretreated with syrosingopine. From Figure 5 it may be seen that any circulating isoprenaline causing an increase in heart rate of less than 450, expressed as $\Sigma\Delta$ HR, would not contribute to the increase seen in untreated mice.

The relationship derived in Figure 6 permits a correction to be applied to the values obtained for the tachycardia due to intracerebroventricular isoprenaline in reserpine-treated mice. In Figure 3 for example, it will be apparent that the degree of tachycardia seen after 5 ng isoprenaline in reserpine-treated mice would have been completely absent in untreated mice if the effects of isoprenaline were entirely peripheral. Thus, for this dose given intracerebroventricularly in such mice, the amount of amine leaking into the circulation is insufficient to contribute to the tachycardia observed in mice not treated with reserpine, which must therefore be of central origin. It is, however, an amount adequate to produce measurable tachycardia in supersensitive mice.

Figure 9 shows, for four doses of isoprenaline given intracerebroventricularly, the tachycardia after various intervals in atropinized mice and in mice additionally pretreated with reserpine, together with the residual values obtained by applying the above correction. It will be seen that only with doses above 5 ng is there any appreciable contribution from circulating amine;

reference to dose-response relations for intravenous isoprenaline suggests that, at peak, this represents no more than 10% of the injected dose. The effect represented by the difference between the dotted and lower unbroken lines in Figure 9 may thus be regarded as of central origin.

These conclusions are supported by the findings in spinal mice, in which intracerebroventricular isoprenaline caused only a slowly developing tachycardia, the initial peak effect being entirely absent (Figure 7). This effect must be attributed solely to a direct action of the amine consequent upon leakage, since all nervously mediated effect of central origin is precluded. It may be seen to resemble the effect remaining in reserpine-treated mice after applying the correction for supersensitivity (compare Figure 9c).

It appears, however, that some degree of supersensitivity is induced even by acute spinalization, since responses to intravenous isoprenaline were significantly greater in spinal mice than in controls (Figure 8). When allowance is made for this in a similar manner to that shown in Figure 6, the residual effects of intracerebroventricular doses of isoprenaline in spinal mice resemble closely those derived from reserpine-treated mice and shown in Figure 9.

Thus, the effects of intracerebroventricularly injected isoprenaline include tachycardia due both to a central action and a peripheral one consequent upon leakage of the amine into the systemic circulation. In this, the situation resembles that reported for noradrenaline by Cowell & Davey (1968) where, however, central and peripheral actions opposed each other whereas those of isoprenaline are similar. Gagnon & Melville (1967) and Bhargava *et al.* (1972) have shown that intracerebroventricular isoprenaline stimulated central β-adrenoceptors since β-adrenoceptor blocking agents injected by the same route blocked the effects. It may be presumed that the central components of the actions of isoprenaline in the mouse are similarly mediated, though leakage of β-adrenoceptor blocking agents after intracerebroventricular injection in mice would preclude a decisive experiment along these lines, since the circulating agent could affect a tachycardia of central origin by blocking peripherally.

The consequences of central β-adrenoceptor stimulation by isoprenaline include hypotension as well as tachycardia (Gagnon & Melville, 1967; Bhargava *et al.*, 1972). Reflex tachycardia evoked by the fall in blood pressure would be nervously mediated and indistinguishable from that directly due to central receptor stimulation by any of the methods used in the work reported here or by the authors quoted.

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EFFECTS OF RESERPINE ON THE ADRENAL MEDULLA OF THE SPONTANEOUSLY HYPERTENSIVE RAT

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1 Reserpine administration resulted in a larger initial decline in adrenal catecholamines in spontaneously hypertensive rats (SHR) than in normotensive Wistar rats (NWR), the difference was eliminated by pretreatment with chlorisondamine.

2 Reserpine also produced a larger increase in SHR catecholamines and dopamine β-hydroxylase several days later; chlorisondamine pretreatment did not prevent the increases, although it did slightly slow the increases.

3 Vesicles from SHR or NWR incubated with reserpine *in vitro* demonstrated equivalent inhibition of adenosine 5′-triphosphate (ATP)-Mg^{2+} -stimulated adrenaline uptake

4 Recovery of uptake was more rapid in SHR than in NWR after reserpine inhibition, and this was associated with a burst of new vesicle synthesis in the SHR; chlorisondamine pretreatment reduced the number of new, immature vesicles in reserpine-treated SHR.

5 Both SHR and NWR secreted equal proportions of their adrenal catecholamine contents after nicotine administration.

6 These data suggest that the sympatho-adrenal system of the SHR exhibits an enhanced reflex response to reserpine but that reserpine is equally effective in SHR and NWR in producing blockade of vesicular catecholamine transport; these alterations can affect markedly the actions of autonomic drugs in the SHR.

Introduction

The sympatho-adrenal axis of the Wistar-derived spontaneously hypertensive rat (SHR) shows marked differences in catecholamine disposition when compared to normotensive Wistar rats (NWR). These include alterations in the levels of catecholamines and the catecholamine-synthesizing enzymes, tyrosine hydroxylase, dopa decarboxylase and dopamine β-hydroxylase (Ozaki, Hotta & Aoki, 1972; Nagatsu, Mizutani, Nagatsu, Umezawa, Matsuzaki & Takeuchi, 1972; Ueba, Mori & Tomomatsu, 1972; Spector, Tarver & Berkowitz, 1972; Lovenberg, Yamabe, deJong & Hansen, 1974), differences in catecholamine turnover (Louis, Spector, Tabei & Sjoerdsma, 1969; Louis, Tabei, Spector & Sjoerdsma, 1969; Nakamura, Gerold & Thoenen, 1972; Spector *et al.*, 1972) and probably a lowering of neural input to the adrenal (Slotkin & Green, 1975). Several of the properties of the catecholamine storage vesicle membrane also appear to be altered: there is less dopamine β-hydroxylase per vesicle and a lowered *Km* for inward transport of adrenaline (Slotkin & Green, 1974). In addition, the SHR exhibits an enhanced catecholamine secretion upon administration of insulin (Slotkin & Green, 1975). It is not clear whether these differences are a causative factor in the development of hypertension, whether they result from the hypertension or whether they represent ancillary strain-dependent factors unrelated to hypertension (Lovenberg *et al.*, 1974). Regardless of the origin of the altered catecholamine disposition, several of the differences could affect the response of the SHR to autonomic agents; for instance, the lower level of dopamine β-hydroxylase (Lovenberg *et al.*, 1974; Slotkin & Green, 1975) renders the SHR more sensitive to inhibitors of that enzyme, and consequently a prolonged decrease in blood pressure (Nagatsu *et al.*, 1972). In the present study, we have examined the effects of reserpine, which evokes a reflex sympatho-adrenal discharge but also causes catecholamine depletion by inhibiting the adenosine 5′-triphosphate (ATP)-Mg^{2+} -dependent transport system in the storage vesicle membrane. Since both neural input to the adrenal and catecholamine uptake into the vesicles

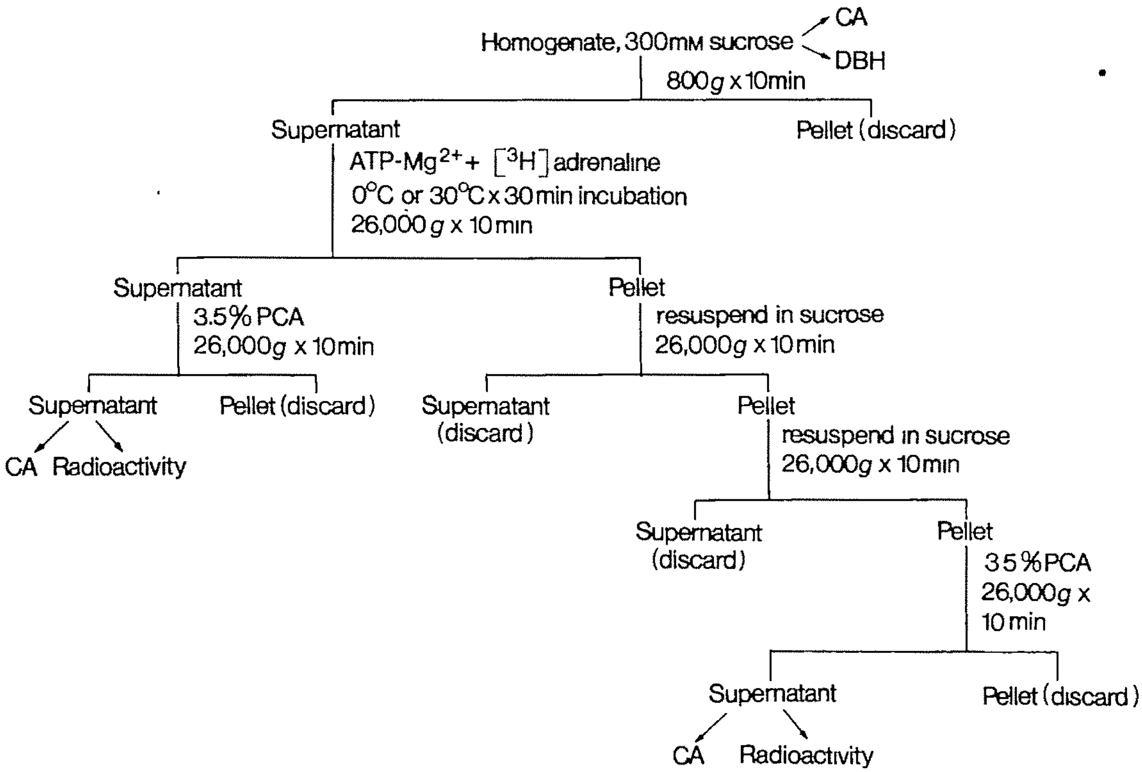

Figure 1 Flow sheet for treatment of rat adrenal homogenates. CA = catecholamine assay, DBH = dopamine β-hydroxylase assay, PCA = perchloric acid.

appear to be altered in the SHR, it is reasonable to expect that the actions of antihypertensive drugs will also be different in the SHR. The present study demonstrates that differences do occur in SHR in the effects of reserpine and the ganglionic blocking agent, chlorisondamine.

Methods

Female normotensive Wistar rats (NWR) and Wistar-derived spontaneously hypertensive rats (SHR) were obtained from Carworth Farms and used at 10-12 weeks of age. Groups of animals were given one of the following treatments: (1) reserpine, 2.5 mg/kg, (2) chlorisondamine, 20 mg/kg, (3) chlorisondamine, 20 mg/kg, followed 1 h later by reserpine, 2.5 mg/kg, or (4) chlorisondamine, 20 mg/kg, followed 1 h later by reserpine, 10 mg/kg; all drugs were injected subcutaneously. The rats were killed 4, 24, 72, 144 or 240 h after reserpine treatment; the adrenal glands from each animal were removed, cleaned of fat and connective tissue, and homogenized in 2.5 ml of sucrose-Tris (300 mM sucrose buffered at pH 7.4 with 25 mM Tris) containing 0.01 mM iproniazid (irreversible monoamine oxidase inhibitor). Aliquots were withdrawn for assay of catecholamines and dopamine β-hydroxylase and the remainder was centrifuged at 800 g for 10 minutes. Aliquots of the supernatant were used for the determination of adrenaline uptake as described previously (Slotkin, 1973); the sucrose incubation medium contained 5 mM ATP, 5 mM Mg^{2+}, 0.1 mM adrenaline and 5μCi [^{3}H]-adrenaline and samples were incubated for 30 min at 30°C while duplicate tubes were kept on ice. Following two washes and centrifugations at 26,000 g of the vesicular pellet, the labelled vesicles were lysed with 3 ml of 3.5% perchloric acid and analyzed for catecholamines and radioactivity. The incubation medium was also analyzed to determine the specific activity. A flow sheet of the procedure appears in Figure 1. The uptake was calculated as described in an earlier communication (Slotkin & Kirshner, 1973a) and the results expressed as uptake per gland and as uptake per 100 μg endogenous catecholamines. The first parameter measures the net uptake capacity of the entire tissue while the latter represents the abilities of individual vesicles to take up amines relative to their catecholamine contents. Under these conditions, the uptake of adrenaline occurs solely into storage vesicles, despite the presence of contaminating particles in the incubation medium (Slotkin & Kirshner, 1971; Slotkin & Kirshner, 1973b).

Studies of the *in vitro* effects of reserpine and harmine on uptake in NWR and SHR adrenal vesicles were conducted in a similar fashion except that animals received no drug treatment; instead the agents were added directly to the incubation medium in concentrations ranging from 2 x 10^{-8} M to 2 x 10^{-6} M for reserpine and from 10^{-6} M to 10^{-4} M for harmine.

Nicotine-induced secretion was determined by the administration of 10 mg/kg subcutaneously; the rats were killed 4 h later. Adrenals were homogenized in 5 ml of 3.5% perchloric acid, centrifuged at 26,000 *g* to remove precipitated protein, and the supernatant analyzed for catecholamines.

Assays

Catecholamines were analyzed by the trihydroxyindole method (without prior adsorption on alumina) using an autoanalyzer and $[^3H]$-adrenaline was determined by liquid scintillation spectrometry (Merrills, 1963; Slotkin, Ferris & Kirshner, 1971). Dopamine β-hydroxylase activity (DBH, E.C. 1.14.2.1) was determined by a modification (Slotkin *et al.*, 1971) of the method of Friedman & Kaufman (1965) using 1 mM parahydroxymercuribenzoate to inactivate endogenous inhibitors (Duch, Viveros & Kirshner, 1968). Incubations were carried out for 1 h at 37°C using 1 μCi $[^3H]$-tyramine (10 μM) as substrate.

Statistical analysis

Data are reported as means ± standard errors and levels of significance determined by Student's *t*-test.

Drugs

Adrenaline-[7-^{3}H] and tyramine-[G-^{3}H] were obtained from New England Nuclear Corporation, chlorisondamine chloride and reserpine phosphate from Ciba Pharmaceuticals, tyramine hydrochloride, parahydroxymercuribenzoate and nicotine from Sigma Chemical Company, and adrenaline bitartrate from Winthrop Laboratories.

Results

Effects of chlorisondamine (Table 1)

Twenty-five hours after the administration of chlorisondamine there were no significant changes in adrenal catecholamines, dopamine β-hydroxylase or in adrenaline uptake in isolated vesicles in either normotensive Wistar rats (NWR) or spontaneously hypertensive rats (SHR).

Effects of reserpine on catecholamines

Four hours after administration of 2.5 mg/kg of reserpine to NWR, there was no change in catecholamines, but levels were decreased to 60% of control by 24 h (Figure 2a). On the other hand, catecholamines in the SHR were markedly lower 4 h after 2.5 mg/kg and were 25% of control by 24 h (Figure 2a). By 3 days post-reserpine, catecholamines had increased nearly to control levels in NWR, but only to 70% in SHR. However

Table 1 Effects of chlorisondamine (20 mg/kg s.c.) on adrenal medullae of normotensive Wistar rats (NWR) and spontaneously hypertensive rats (SHR).

Rat strain	*Treatment*	*Catecholamines (μg/gland)*	*Dopamine β-hydroxylase (nmol/h per gland)*	*Adrenaline uptake*		*Number of animals*
				nmol per gland	*nmol per 100 μg catecholamines*	
NWR	None	15.0 ± 0.5*	1.57 ± 0.05	3.04 ± 0.13	20.5 ± 1.0	37
	25 h after chlorisondamine	14.5 ± 0.2	1.70 ± 0.08	3.07 ± 0.12	21.3 ± 0.8	5
SHR	None	11.2 ± 0.4	0.69 ± 0.02	2.74 ± 0.08	24.6 ± 1.8	39
	25 h after chlorisondamine	10.4 ± 0.3	0.73 ± 0.05	2.74 ± 0.14	26.1 ± 0.5	7

*Mean ± s.e.

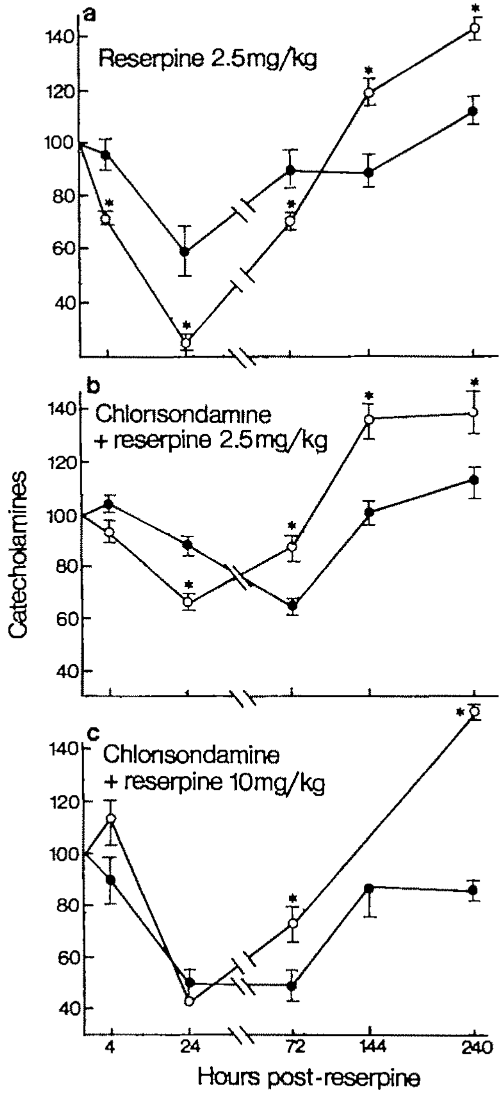

Figure 2 Effects of reserpine on adrenal catecholamines in normotensive Wistar rats (NWR) (●) and spontaneously hypertensive rats (SHR) (○) In (b) and (c), chlorisondamine (20 mg/kg) was administered 1 h before reserpine. Each point represents mean of 5-6 animals in terms of percent of control; vertical bars show s.e. mean. Control values are given in Table 1.
* Significant differences ($P < 0.05$ or better) between SHR and NWR values.

6 and 10 days post-reserpine SHR catecholamines were substantially elevated above control while NWR levels were normal.

When chlorisondamine was administered prior to reserpine (2.5 mg/kg), the initial (4 h) decline in SHR catecholamines seen with reserpine alone did not occur (Figure 2b). Furthermore, the degree of depletion after 24 h was less in both NWR and SHR than after reserpine alone, although SHR were still depleted to a greater extent than NWR at this time (Figure 2b). Three days after reserpine and chlorisondamine, SHR catecholamines were 90% of controls but NWR were only 65%; at subsequent times, the levels in SHR were substantially supranormal while NWR were at or slightly above control (Figure 2b).

Chlorisondamine pretreatment also prevented any initial (4 h) catecholamine-depleting effect of high doses of reserpine (Figure 2c). Twenty-four hours later, both NWR and SHR were depleted to 40-50% of control catecholamine levels. NWR catecholamines remained depleted between 1 and 3 days post-reserpine and increased toward control levels by 6-10 days, while in the SHR some recovery was evident by 3 days and catecholamines were 150% of controls by 10 days (Figure 2c).

Effects of reserpine on dopamine β-hydroxylase (DBH) activity

Administration of reserpine (2.5 mg/kg) alone produced a small decline in DBH in NWR and SHR at 4 and 24 h, but an increase at subsequent time periods (Figure 3a). The elevation was much more pronounced in SHR than in NWR.

Chlorisondamine pretreatment prevented the initial reserpine-induced decline in DBH at 4 and 24 h, and in fact resulted in a significant increase in DBH in SHR at 24 h (Figure 3b); subsequent increases in DBH of NWR and SHR equalled or exceeded those seen at 3, 6 and 10 days after reserpine alone (Figure 3b). A similar pattern of change in DBH was seen after chlorisondamine + 10 mg/kg of reserpine (Figure 3c).

Effects of reserpine on adrenaline uptake per gland

Reserpine (2.5 mg/kg) produced nearly total blockade of adrenaline uptake per gland in isolated storage vesicles in both NWR and SHR at 4 h (Figure 4a). There was only slight recovery at 24 h, but by 3 days, uptake per gland was normal in NWR and supranormal in SHR; uptake per gland remained elevated in SHR up to 10 days post-reserpine (Figure 4a).

Chlorisondamine pretreatment did not alter the reserpine-induced decrease in adrenaline uptake per gland at 4 or 24 h but appeared to slow the recovery in both NWR and SHR (Figure 4b). Uptake per gland reached control levels by 6 days post-reserpine and, in SHR, exceeded control levels at 10 days (Figure 4b). Similar results were obtained with chlorisondamine followed by 10 mg/kg of reserpine (Figure 4c).

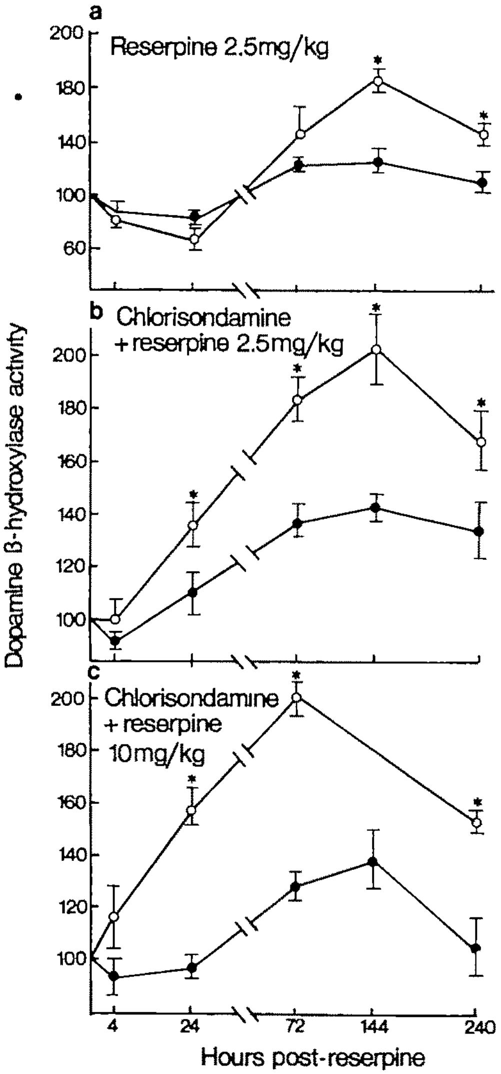

Figure 3 Effects of reserpine on adrenal dopamine β-hydroxylase activities in normotensive Wistar rats (NWR) (●) and spontaneously hypertensive rats (SHR) (○). In (b) and (c), chlorisondamine (20 mg/kg) was administered 1 h before reserpine Each point represents mean of 5-6 animals in terms of percent of control; vertical bars show s.e. mean. Control values are given in Table 1.

* Significant differences ($P < 0.05$ or better) between SHR and NWR values. Vertical scale is compressed in (a).

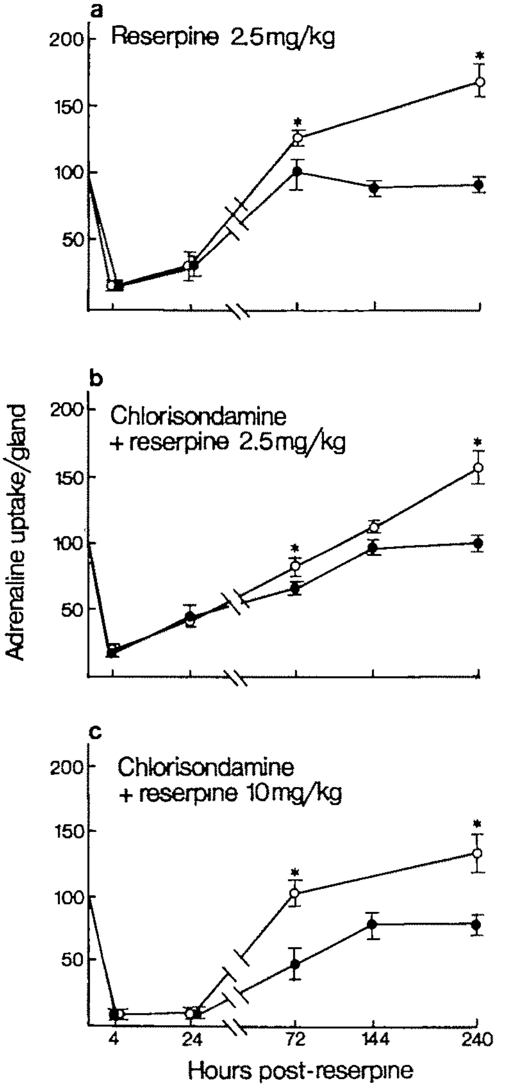

Figure 4 Effects of reserpine on [^{3}H]-adrenaline uptake per gland in isolated adrenal storage vesicles from normotensive Wistar rats (NWR) (●) and spontaneously hypertensive rats (SHR) (○). In (b) and (c), chlorisondamine (20 mg/kg) was administered 1 h before reserpine. Each point represents mean of 5-6 animals in terms of percent of control; vertical bars show s.e. mean. Control values are given in Table 1.

* Significant differences ($P < 0.05$ or better) between SHR and NWR values.

Effects of reserpine on adrenaline uptake per 100 µg endogenous catecholamines

Reserpine (2.5 mg/kg) alone reduced uptake/100 µg catecholamines to about 20% of controls at 4 h in both NWR and SHR (Figure 5a). However, by 24 h NWR values had returned to 50% of control and SHR uptakes were elevated above controls. At subsequent times, uptakes/100 µg catecholamines were at or near control levels for NWR; in SHR, the highest value (>170% of control) was reached at 3 days and declined to 115% by 10 days post-reserpine (Figure 5a).

Chlorisondamine pretreatment did not alter the

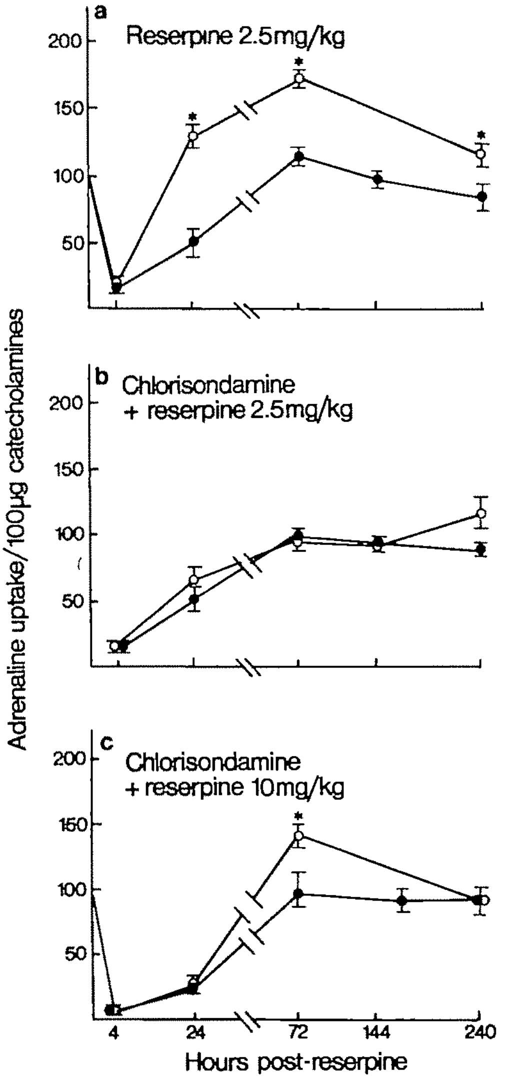

Figure 5 Effects of reserpine on [^{3}H]-adrenaline uptake per 100 μg of endogenous catecholamines in isolated adrenal storage vesicles from normotensive Wistar rats (NWR) (●) and spontaneously hypertensive rats (SHR) (○). In (b) and (c), chlorisondamine (20 mg/kg) was administered 1 h before reserpine. Each point represents mean of 5-6 animals in terms of percent of control; vertical bars show s.e. mean. Control values are given in Table 1.
* Significant differences ($P < 0.05$ or better) between SHR and NWR values.

initial effect of the low dose of reserpine on uptake/100 μg catecholamines in NWR but prevented the later increases which had been seen in SHR after reserpine alone (Figure 5b). There was no difference between NWR and SHR in the inhibition of uptake and subsequent recovery with chlorisondamine + 2.5 mg/kg of reserpine (Figure 5b). After chlorisondamine + 10 mg/kg of reserpine, the decrease in uptake/100 μg catecholamines in NWR and SHR was more profound at 4 and 24 h than at the lower dose (Figure 5c). NWR recovered to control levels by 3 days, while SHR exceeded control at 3 days and returned to normal by 10 days (Figure 5c).

Effects of reserpine and harmine in vitro *on adrenaline uptake per 100 μg endogenous catecholamines*

Reserpine and harmine *in vitro* inhibited adrenaline uptake into isolated storage vesicles to approximately the same extent in NWR and SHR. The calculated IC$_{50}$'s (8 determinations) for reserpine were 0.11 ± 0.02 μM in NWR vesicles and 0.13 ± 0.02 μM in SHR vesicles; for harmine, IC$_{50}$'s were 20 ± 5 μM in NWR vesicles and 12 ± 2 μM in SHR vesicles.

Nicotine-induced catecholamine secretion

Four hours after the administration of nicotine, adrenal catecholamine levels had decreased to 79 ± 2% of control in NWR (14 animals); and to 78 ± 2% of control in SHR (12 animals).

Discussion

The effects of reserpine on the adrenal medulla consist of three distinct actions: an initial reflex stimulation mediated by the splanchnic nerve and resulting in depletion of catecholamine stores *via* exocytotic, all-or-none release of the soluble content of the storage vesicles; a further depletion of catecholamines due to blockade of the ATP-Mg^{2+}-dependent uptake system in the storage vesicle membrane; and a later, less intense reflex stimulation in response to the hypotension which results from sympathetic catecholamine depletion. Thus, in comparing the reserpine-induced alterations in catecholamine disposition in normotensive Wistar rats (NWR) and spontaneously hypertensive rats (SHR) it is important to establish which differences are attributable to direct, and which to reflex, effects of the drug.

A recent study (Slotkin & Green, 1975) demonstrated that the vesicular uptake system of the SHR exhibited a lower *Km* for adrenaline than did that of NWR, indicating an enhanced uptake of catecholamines in the SHR. In the current experiments, two competitive inhibitors (reserpine and harmine) of the uptake system (Jonasson, Rosengren & Waldeck, 1964; Green & Slotkin, 1973) were just as effective *in vitro* in SHR vesicles as in NWR vesicles. These data suggest that the *Ki* for the inhibitors is reduced in the SHR in a fashion similar to the reduction in the *Km* for adrenaline, and that reserpine is therefore equally effective in its direct effect in SHR and NWR. The *in vivo* studies confirm this hypothesis: in all cases, the inhibition of adrenaline uptake per gland or per 100 μg endogenous catecholamines was equivalent in SHR and NWR at 4 h post-reserpine. Consequently, the differences seen between SHR and NWR in the effects of reserpine *in vivo* probably result primarily from differences in reflex stimulation of the splanchnic nerve.

Low doses (2.5 mg/kg) of reserpine elicited little or no initial reflex secretion in NWR, as indicated by the absence of a significant decrease in catecholamines at 4 hours. In contrast, there was a prompt decline in SHR catecholamine levels. To test whether this was due to a greater reflex effect in the SHR, another group of rats was pretreated with chlorisondamine, which blocks splanchnic stimulation of the adrenal medulla; under these conditions reserpine failed to produce depletion in SHR at 4 h, indicating that the SHR does exhibit a greater neural reflex effect to reserpine alone than the NWR. Similar results have been obtained with insulin (Slotkin & Green, 1975), which also elicits reflex activity, suggesting that the exaggerated sympatho-adrenal response in the SHR is a general phenomenon and is not specific only to reserpine. To determine whether this property is due to a difference in the adrenal medulla itself or rather to a more proximal alteration in the reflex pathway, NWR and SHR were given nicotine, which stimulates the adrenal medulla directly. Both NWR and SHR secreted equal proportions of their catecholamine stores, indicating that the adrenal glands themselves were equally responsive in the two strains. These data suggest that the site of the alteration may be in central regulation of autonomic reflexes.

If the SHR is in fact abnormally responsive to reserpine-induced stimulation, then other biochemical parameters which are dependent on neural input should also demonstrate differences from NWR. Thus, there was a greater induction of dopamine β-hydroxylase in SHR 6-10 days after reserpine alone than in NWR, indicating a more rapid synthesis of new storage vesicles (Slotkin & Kirshner, 1973b). This explains in part the large increase in adrenaline uptake per gland in SHR 3-10 days after reserpine: despite the greater loss of vesicles from neurogenic secretion, the further increment in neural input from reserpine and the resultant hypotension produces a more rapid vesicle synthesis. The large burst of new synthesis results in the formation of 'immature' vesicles (Slotkin & Kirshner, 1973a,b; Slotkin, 1973) with low catecholamine to dopamine β-hydroxylase ratios (Figures 2a and 3a) and consequently an increased adrenaline uptake per 100 μg endogenous catecholamines (Figure 5a). Thus, reserpine alone in the SHR causes a greater initial depletion of catecholamines and storage vesicles, and a higher degree of resynthesis at subsequent times.

The data obtained with chlorisondamine-pretreated animals tend to support the hypothesis that stimulation is more intense in the SHR in both initial and late time periods. While ganglionic blockade prevented the initially (4 h) greater depletion of SHR catecholamines, there was still a significant difference between SHR and NWR at 24 h; since chlorisondamine is a competitive antagonist, it is likely that the increased splanchnic stimulation in the SHR results in overcoming of the blockade at an earlier point than in NWR. The prevention by chlorisondamine of the reserpine-induced initial reflex discharge led to a slowing of the depletion phase but also to a slower rate of subsequent increase in catecholamines: reserpine-treated SHR increased by 20% between days 1 and 3 with chlorisondamine pretreatment, but by 45% with reserpine alone; the 25% increase seen in NWR given reserpine alone was reversed (25% decline) with chlorisondamine-pretreatment. Inhibition of stimulation similarly slowed the rate of recovery of adrenaline uptake per gland, suggesting that the large burst of new vesicle synthesis in SHR had been reduced; this was confirmed by the absence of 'immature' vesicles as indicated by a normal adrenaline uptake per 100 μg endogenous catecholamines.

The apparent facilitation by chlorisondamine of the reserpine-induced increases in dopamine β-hydroxylase activity in both SHR and NWR do not indicate a larger degree of induction: the prevention of the initial secretion results in the preservation of a larger number of intact old vesicles, the membranes of which would otherwise have been destroyed or recycled 1 to 2 days after secretion (Slotkin & Kirshner, 1973b). Under these conditions, adrenaline uptake per 100 μg endogenous catecholamines is a better measure of new vesicle formation than is enzyme activity.

The administration of larger doses of reserpine after chlorisondamine resulted in an accentuation of differences between SHR and NWR which could be attributed to a more intense late stimulatory phase in the SHR. The disparities between SHR and NWR catecholamines, dopamine β-hydroxylase and adrenaline uptake per gland were larger and the differences in adrenaline uptake per 100 μg catecholamines suggested that immature vesicles were present at 3 days in the SHR. However, there were fewer immature vesicles present in SHR given chlorisondamine and

10 mg/kg reserpine than after 2.5 mg/kg reserpine alone, indicating the importance of the initial stimulation for the large burst of new vesicle synthesis.

In conclusion, although the basal sympatho-adrenal tone of the SHR appears to be lower than that of the NWR, the SHR exhibits an apparent hyper-reactivity in response to drugs which elicit sympatho-adrenal reflexes. Consequently, considerable caution must be exercised in evaluating the activities of autonomic agents in the SHR.

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NICOTINE-INDUCED STIMULATION OF STEROIDOGENESIS IN ADRENOCORTICAL CELLS OF THE CAT

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1 The effect of nicotine on steroid production and release from trypsin-dispersed cat adrenocortical cells was investigated.

2 Nicotine, like adrenocorticotrophin (ACTH), elicited a dose-dependent increase in steroidogenesis, which depended upon the presence of calcium in the medium.

3 Augmented steroid production evoked by submaximal concentrations of ACTH, monobutyryl cyclic adenosine 3',5'-monophosphate (AMP), or prostaglandin E_2 was further enhanced by steroidogenic concentrations of nicotine.

4 These results are discussed in relation to the possible mode of action of nicotine on cortical cells and to the potential consequences of smoking during stress.

Introduction

The diverse pharmacological effects of nicotine on biological systems have been most extensively studied in electrically excitable tissues such as nerve and muscle. The depolarization of autonomic ganglia is the most characteristic action of nicotine (Paton & Perry, 1953), and related to this effect, nicotine augments catecholamine release from the homologous chemosensitive adrenomedullary chromaffin cells by promoting an influx of extracellular calcium (Douglas & Rubin, 1961; Rubin & Miele, 1968). The depolarizing activity of nicotine extends to nerve terminals where it enhances neurotransmitter release (Lee & Shideman, 1959; Bhagat, 1966) and has been reported to augment vasopressin release from neurosecretory nerve terminals in the neurohypophysis (Burn, Truelove & Burn, 1945; Beardwell, 1971), although this latter finding was not corroborated *in vitro* (Thorn, 1966).

In contrast to the existing evidence regarding the action of nicotine on secretory organs of neuronal origin, much less is known of the effects of nicotine on secretory tissues of non-neuronal origin. The present studies focus on the action of nicotine on cells of the adrenal cortex. Knowledge of the effects of this alkaloid on the gland which plays such a key role in the organism's response to stress is of considerable interest and importance in the light of its widespread use.

Although recent work done in our laboratory using the *in situ* perfused cat adrenal gland has yielded a great deal of evidence concerning the mechanism of ACTH action (Jaanus, Rosenstein & Rubin, 1970; Jaanus & Rubin, 1971, Carchman, Jaanus & Rubin, 1971; Jaanus, Carchman & Rubin, 1972), the cat isolated adrenocortical cell preparation was selected for this investigation because of the homogeneity of its cell population and the ability to obtain readily dose-response relationships which cannot be so easily accomplished with the intact gland (Jaanus, *et al.*, 1970).

Methods

Preparation of the cortical cell suspension

Trypsin-dispersed cat adrenocortical cells were prepared according to a modification of the method of Sayers, Swallow & Giordano (1971) for dispersing rat adrenocortical cells. Male cats were anaesthetized with an intraperitoneal injection of pentobarbitone sodium (50 mg/kg). The adrenal glands were quickly removed and placed in cold modified Krebs solution, containing Eagle's Minimal Essential Medium (MEM) plus glutamine (2 mM). The Krebs MEM was equilibrated with a gas mixture of 95% O_2 and 5% CO_2 and had a pH of 7.4. The glands were cut into $8\ \mathrm{mm}^3$ cubes, placed in siliconized trypsinizing flasks containing 10 ml Krebs MEM plus trypsin (0.5%) and bovine serum albumin (0.25%), and incubated at room temperature in a metabolic shaker. After 90 min, the temperature was raised to 37° C, and the rate of shaking increased from 120-400 rev/minute. After 1 h the undigested material was removed and the suspension incubated for an additional 20 min at 120 rev/min with sufficient trypsin inhibitor to block further proteolytic activity. The amount of inhibitor added depended upon the quantity of trypsin present and the activity of the trypsin inhibitor. The cell suspension was washed once with Krebs MEM and finally resuspended in Krebs MEM containing bovine serum albumin (0.5%) and trypsin inhibitor (0.2%).

Two types of cortical cells could be distinguished microscopically, larger cells (17 μm in diameter) with densely speckled cytoplasm and slightly smaller cells (15 μm) with a lighter appearance. The cortical cells were about four times larger and had a darker cytoplasm than erythrocytes, which were the major contaminant in the system. Contamination by other tissue components, such as chromaffin cells, adipose tissue and cell fragments, was minimized since they were either digested by the trypsin (Malamed, Sayers & Swallow, 1970) or they remained suspended during centrifugation, while the cortical cells formed a pellet. The cortical cells were counted with a haemocytometer and the cell concentration adjusted to approximately 250,000 cells per ml (equivalent to the final incubation volume). The cells were incubated at 37° C for 2 h in normal or modified Krebs MEM in the absence or presence of stimulating agent. At the end of the incubation, 1 ml of Krebs MEM was added to rinse the beakers, and the suspension was centrifuged to separate the cells from the supernatant.

Steroid analysis

Corticosteroids, mainly in the form of hydrocortisone, were extracted from the supernatant with 5 ml methylene chloride and assayed by competitive protein binding using transcortin from human plasma as the binding agent (Jaanus *et al.*, 1972). The calculated steroid values were expressed either in ng/250,000 cells per 2 h ($\mathrm{ng\ ml^{-1}\ 2\ h^{-1}}$) or as percent increase relative to unstimulated control cells.

Drugs and reagents used

Trypsin and lima bean trypsin inhibitor, (Worthington); bovine serum albumin and minimal Eagle's medium, (Schwarz-Mann); monobutyryl cyclic adenosine 3′,5′-monophosphate (AMP), (Sigma); nicotine base, (Eastman); $[^3\mathrm{H}]$-corticosterone (42 Ci/mM), (New England Nuclear); β 1-24 adrenocorticotrophin (ACTH) (Ciba); prostaglandin E_2 (Upjohn) and NPS-ACTH (from Dr J. Ramachandran, University of California at San Francisco).

Results

Basal and ACTH-stimulated steroid levels

Cells incubated in Krebs solution for 2 h in the absence of ACTH manifested a fairly constant, low level of steroid production and release, with an average of 32 $\mathrm{ng\ ml^{-1}\ 2\ h^{-1}}$ (Table 1). The addition of microunit concentrations of ACTH to the medium for 2 h caused a dose-related increase in steroidogenesis (Figure 1). Although responsiveness to ACTH varied from preparation to preparation, ACTH concentrations of 1-2 μu/ml generally produced discernible increases in steroid production. The ED_{50} appeared to be approximately 50 μu/ml and a plateau was achieved at or above 500 μu/ml.

Effects of nicotine on steroid production

The stimulant effect of various nicotine concentrations on basal steroid production is shown in Table 1. Nicotine, in a concentration of 6 μM, produced a small but inconsistent increase in steroid production, whereas 60 μM nicotine

Table 1 Effect of nicotine on basal and adrenocorticotrophin (ACTH)-induced steroid release from isolated adrenocortical cells

Experiment	*No. of experiments*	*Steroid release ($\mathrm{ng\ ml^{-1}\ 2\ h^{-1}}$)*
Control	11	32 ± 3.5
Nicotine		
6 μM	4	35 ± 7.0
60 μM	9	53 ± 8.0[a]
600 μM	9	79 ± 8.0[a]
ACTH 25 μu/ml	9	77 ± 9.0
ACTH + nicotine		
6 μM	4	83 ± 17
60 μM	10	110 ± 12[b]
600 μM	9	121 ± 16[b]

[a] Significantly greater than control by Student's *t* test ($P < 0.05$).

[b] Significantly greater than ACTH control by Student's *t* test ($P < 0.05$).

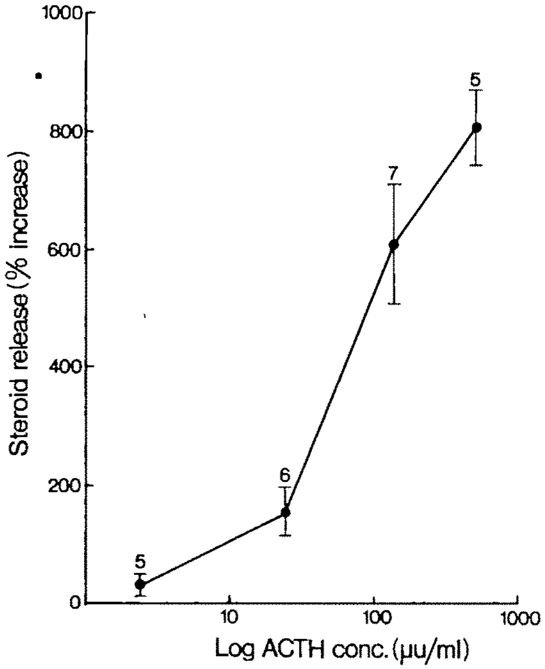

Figure 1 Dose-response relationship of the steroidogenic effect of adrenocorticotrophin (ACTH) on trypsin-dispersed cat adrenocortical cells. Following trypsinization (see methods section) the cells were incubated for 2 h in fortified Krebs solution which also contained 0.5% bovine serum albumin, 0.2% lima bean trypsin inhibitor, plus varying concentrations of ACTH. The cells were centrifuged and the supernatant assayed for steroid. The values represent average steroid production calculated from the number of experiments indicated in parentheses. Vertical bars show s.e. mean.

augmented steroid production by more than 65%. A 150% augmentation in steroid production was observed with the highest nicotine concentration used (600 μM). Higher nicotine concentrations were not tested due to the potential danger of alterations in the pH of the medium which might be produced by the alkaloid.

When nicotine was added to the medium containing a submaximal concentration of ACTH (25 μu/ml), an additional increase in steroid release was observed (Table 1). However, the increase produced by nicotine in the presence of ACTH was clearly observed only at those concentrations (60 and 600 μM) which by themselves were steroidogenic. Thus, ACTH, in the absence and presence of 6 μM nicotine increased steroidogenesis by 140 and 160%, respectively; whereas ACTH plus nicotine 60 and 600 μM increased steroidogenesis by 3-4 fold, 250 and 278%, respectively (Table 1).

Additional insight into the nature of the interaction between ACTH and nicotine was obtained in an experiment in which a particular cell preparation was quite unresponsive to ACTH (25 μu/ml), steroidogenesis increasing only from 50 to 53 ng/ml. In this preparation steroid production elicited by 600 μM nicotine in the absence and presence of ACTH was 130 and 110 ng/ml, respectively. In another experiment when a threshold concentration (250 μu/ml) of the weak agonist, NPS-ACTH, failed to increase steroid production above the basal level of 19 ng/ml, steroidogenesis was increased by nicotine (600 μM) to 64 and 53 ng/ml in the absence and presence of NPS-ACTH, respectively.

Interaction between nicotine and cyclic AMP and prostaglandin E_2.

Cyclic AMP and prostaglandins are two putative mediators of ACTH-induced steroidogenesis; therefore, the effect of nicotine on the steroidogenic action of these agents was also tested. Monobutyryl cyclic AMP was used in place of its parent compound because its greater lipid solubility may enhance its penetration into cells. Prostaglandin E_2 was used since it was the most potent prostaglandin in augmenting steroid production in rat adrenal tissue (Flack, Jessup & Ramwell, 1969).

Although complete dose-response relationships were not ascertained in the present study, both monobutyryl cyclic AMP and prostaglandin E_2 were observed to have similar threshold (0.02-0.05 mM) and plateau (0.4-0.5 mM) concentrations. To test the effects of nicotine on the steroidogenic responses to these two agents, concentrations of the cyclic nucleotide (0.25 mM) and prostaglandin E_2 (0.1 mM) were selected which enhanced steroid production to approximately the same extent as 25 μu/ml ACTH. Table 2 shows that both the lower and higher concentrations of nicotine greatly enhanced the steroidogenic action of butyryl cyclic AMP, but only the higher alkaloid concentration clearly enhanced the action of prostaglandin E_2.

The effect of calcium deprivation on nicotine induced steroidogenesis

The steroidogenic action of ACTH in the intact perfused cat adrenal gland requires the presence of calcium (Jaanus *et al.*, 1970). Similarly, incubation of isolated adrenocortical cells of the cat in a calcium-deprived medium containing (ethylenebis-(oxyethylenenitrilo))-tetraacetic acid (EGTA)

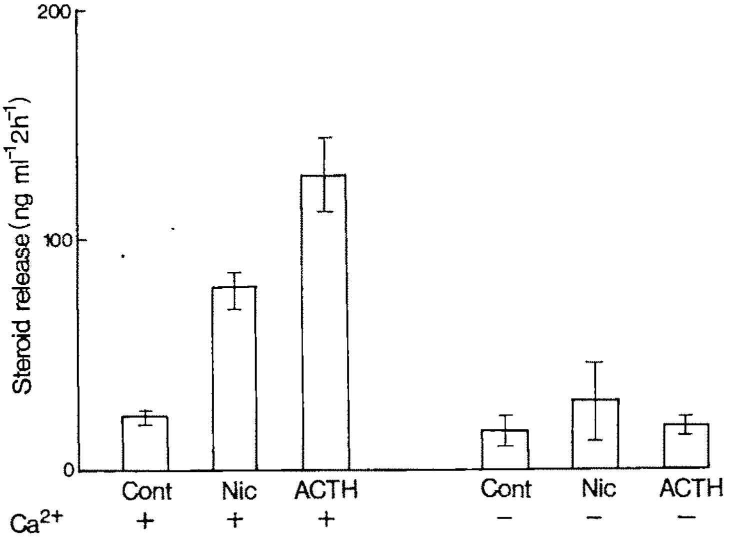

Figure 2 The effect of calcium deprivation on the steroidogenic response to adrenocorticotrophin (ACTH) and nicotine (Nic). Equal numbers of cells were incubated in the normal incubation medium (see methods section), or in calcium-free medium plus EGTA (1 mM) with ACTH (500 µu/ml) or nicotine (600 µM). The columns represent mean steroid values, calculated from at least three experiments. Vertical bars show s.e. mean.

(1 mM), although not discernibly affecting basal steroid production, completely depressed the steroidogenic response to a maximum stimulating ACTH concentration (500 µ/ml) (Figure 2). Steroid production induced by a high nicotine concentration (600 µM) was depressed by calcium deprivation by 70% (Figure 2). In these experiments the average steroid production of 96 ng/ml elicited by nicotine was reduced to 29 ng/ml in the same cell preparations after calcium deprivation.

Table 2 Effect of nicotine on steroid release induced by monobutyryl cyclic AMP (bcAMP) or prostaglandin E_2 (PGE_2)

Experiment	*No. of experiments*	*Steroid release (% increase)*
PGE_2	4	78 ± 8
PGE_2 + nicotine		
60 µM	3	88 ± 38
600 µM	4	408 ± 137
bcAMP	4	90 ± 38
bcAMP + nicotine		
60 µM	3	291 ± 38
600 µM	3	508 ± 185

Mean values with s.e. mean are expressed as % increase over the basal value of a given experiment.

Discussion

Previous investigators have observed, both in experimental animals and in human subjects, a rise in plasma corticosteroids after the administration of nicotine (see, Larson & Silvette, 1971); this stimulation of adrenocortical activity was ascribed to an indirect action resulting from enhanced ACTH release from the adenohypophysis (Kershbaum, Pappajohn, Bellet, Hirabayashi & Shafiha, 1968; Suzuki, Ikeda & Narita, 1973).

The present studies have clearly shown that nicotine is capable of directly stimulating isolated adrenocortical cells of the cat. The stimulant effect is observable in micromolar concentrations, is dose-dependent and like the physiological stimulus, ACTH, depends upon the presence of calcium. Not only is nicotine able to stimulate cortical cells directly but it enhances the steroidogenic effect of ACTH. The interaction between nicotine and ACTH appears to be additive rather than synergistic. This conclusion is based upon the fact that the steroid reponse to ACTH plus nicotine approached the sum of their separate responses. Moreover, nicotine was unable to potentiate a threshold response to ACTH or to NPS-ACTH, a weak ACTH analogue.

The mechanism by which nicotine causes the cortical cells to produce and release steroids cannot be defined at present. It is clear, however, that the stimulation of cortical cells by nicotine, like its action on medullary chromaffin cells (Douglas & Rubin, 1961) requires the presence of calcium. Nicotine, like acetylcholine, appears to stimulate the medulla to secrete by depolarizing the chromaffin cell membrane (Douglas, Kanno & Sampson, 1967) allowing the transmembrane flux of calcium, which in turn triggers the release of preformed catecholamine stored in secretory granules (Douglas, 1968, Rubin, 1970).

However, one must be circumspect about extending the sequence of events thought to exist in the medulla to the cortex; for there appears to be no correlation between steroidogenic activity and the depolarization of cortical cells (Matthews & Saffran, 1967; Jaanus *et al.*, 1970). Moreover, in contrast to the medulla where transmembrane calcium flux is responsible for initiating evoked secretion, in the cortex stimulation by ACTH appears to trigger a cellular translocation of calcium (Jaanus & Rubin, 1971).

Thus, on the basis of these data it is premature and perhaps misleading to ascribe the stimulant effect of nicotine to a membrane depolarizing action on cortical cells. Nicotine can traverse the cell membranes of secretory tissue by a passive diffusion process (Putney & Borzelleca, 1972) and in skeletal muscle may release calcium from cellular sites (Weiss, 1968), so that an intracellular mobilization of calcium could be responsible for nicotine-induced steroidogenesis.

Although calcium has a key role in the stimulant action of nicotine, other proposed modulators of steroidogenesis such as cyclic AMP and prostaglandins may also participate in the events culminating in steroid production and release. The observations that nicotine enhances the steroidogenic action of exogenous cyclic nucleotide and prostaglandin E_2, just as it enhances the action of ACTH, might suggest that these proposed mediators indeed play key roles; however, more conclusive evidence implicating cyclic AMP and/or prostaglandins in nicotine-induced steroid production would be the detection of changes in tissue levels of these proposed mediators during stimulation. These experiments are now in progress.

In regard to the obvious practical implications of the present findings, the ability of nicotine to enhance corticosteroid output may be salutary or detrimental. In individuals having an inadequate response to stress, smoking may enhance their ability to cope with stressful situations by augmenting steroid output. Conversely, nicotine-induced stimulation of cortical cells may cause aberrations in the metabolic and electrolyte balance of the organism, especially in stressful situations where steroid levels are already high.

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THE IRREVERSIBLE BINDING OF ACETYLCHOLINE MUSTARD TO MUSCARINIC RECEPTORS IN INTESTINAL SMOOTH MUSCLE OF THE GUINEA-PIG

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1 Acetylcholine mustard (*N*-2-chloroethyl-*N*-methyl-2-acetoxyethylamine), a potent muscarinic agonist, binds virtually irreversibly to muscarinic receptors in longitudinal muscle strips from guinea-pig small intestine, as shown by the inhibition of the binding of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM), an affinity label for the muscarinic receptor.

2 A value for the apparent binding affinity of acetylcholine mustard and a value for the rate constant for the receptor alkylation reaction have been deduced from the rate of onset of the inhibition of [^{3}H]-PrBCM binding.

3 The kinetic constants obtained may refer largely to the interaction between acetylcholine mustard and the desensitized receptor.

4 At high concentrations acetylcholine mustard practically abolishes the contractile response to carbachol. At these concentrations acetylcholine mustard appears to have multiple actions on the tissue.

Introduction

The formation of a reversible complex between an agonist and its receptor is considered to be the first event in the chain which leads to the physiological response, but until recently methods for obtaining agonist binding affinities in intact tissues have been limited to indirect organ bath techniques (see e.g. Parker, 1972). The development of affinity labels for drug receptors has opened the way for more direct studies of agonist binding via the inhibition by the agonist of the binding of the affinity label. In the special case of an agonist which also binds irreversibly to the receptor there is in principle an alternative approach to the binding affinity from measurements of the rate of formation of the covalently-bound complex and in this paper we describe the application of this method to study the binding of acetylcholine mustard (*N*-2-chloroethyl-*N*-methyl-2-acetoxyethylamine) (Hanby & Rydon, 1947) to muscarinic receptors in longitudinal muscle strips from guinea-pig small intestine.

Acetylcholine mustard in its cyclized aziridinium ion form (Figure 1) is a potent agonist at muscarinic receptors in guinea-pig ileum (Hirst & Jackson, 1972) and rat jejunum (Hudgins & Stubbins, 1972) but, surprisingly, has been reported as showing either no (Hirst & Jackson, 1972) or relatively weak (Hudgins & Stubbins, 1972) irreversible binding. There are, however, experimental difficulties involved in using the contractile response as a measure of the irreversible binding and to circumvent these we have compared the binding of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM), a specific and virtually irreversible muscarinic antagonist (Burgen, Hiley & Young, 1974a) before and after exposure of the tissue to acetylcholine mustard. By this method it is shown that acetylcholine mustard does bind irreversibly to muscarinic receptors in the longitudinal muscle from guinea-pig small intestine and that the rate of onset of block can be used to derive kinetic constants.

Some of these results have been communicated in preliminary form to the British Pharmacological Society (Robinson, Taylor & Young, 1974).

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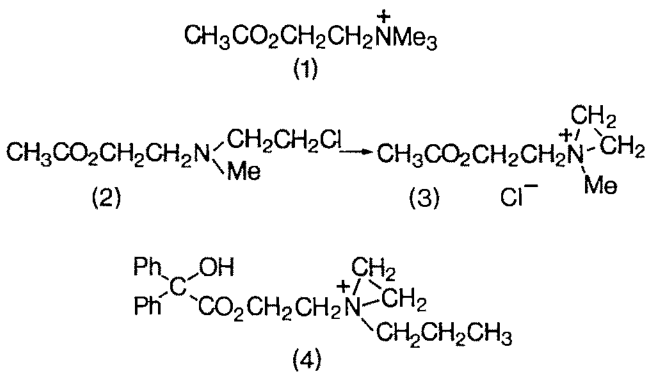

Figure 1 Structures of acetylcholine (1), acetylcholine mustard (2) and its aziridinium ion derivative (3), and the aziridinium ion from propylbenzilylcholine mustard (4).

Methods

Acetylcholine mustard

Acetylcholine mustard was synthesized essentially as described by Hanby & Rydon (1947) and had b.p. 60-62° C/0.2 mm. The n.m.r. spectrum (in $CDCl_3$, measured on a Varian T-60A spectrometer at 60 MHz) was virtually identical with that reported by Hirst & Jackson (1972) for the pure liquid. Assignment of the line positions, made by comparison of the spectrum with that of mustine (see below), measurement of coupling constants and sequential spin-decoupling of the methylene resonances, provided proof of the structure. Assignments (line positions are given in parts/10^6 downfield from tetramethylsilane as internal standard): C-Me, 2.07, s; N-Me, 2.39, s; acetoxyethyl N-CH_2, 2.74, t (J 6.0 Hz); chloroethyl N-CH_2, 2.81, t (J 6.7 Hz); Cl-CH_2, 3.57, t (J 6.7 Hz) and O-CH_2, 4.18, t (J 6.0 Hz).

Further structural evidence was provided by the dimerization of acetylcholine mustard in ethanol to give *N,N'*-dimethyl-*N,N'*-di(2-acetoxyethyl)-piperazinium dichloride, m.p. 221-222° C, literature m.p. 228° C (Hanby & Rydon, 1947), whose n.m.r. spectrum was identical with that given by Hirst & Jackson (1972).

The parent chloroethylamine-compound was converted into the active aziridinium ion form before use by maintaining a 0.7 mM solution in Krebs-Henseleit solution for 45 min at 30° C at which time the yield of the aziridinium ion, 70.5 ± 1% (4 measurements), is maximal. The cyclized solution was used immediately after preparation. *All concentrations of acetylcholine mustard quoted are those of the aziridinium ion form, calculated assuming a 71% yield.* Cyclization was carried out in Krebs solution to avoid any dilution of the Krebs solution constituents when large volumes of the mustard solution had to be added to the incubation medium, but a higher yield of the aziridinium ion (79 ± 0.5%, 3 measurements) can be achieved if cyclization is allowed to proceed in 10 mM phosphate buffer, pH 7.5. The decay of the aziridinium ion concentration, presumably due to hydrolysis, followed first order kinetics, the rate of the decay in Krebs solution at 30°C ($k = 2.3 \pm 0.2 \times 10^{-5}\ s^{-1}$, 4 measurements) being notably slower than in 10 mM phosphate buffer, pH 7:5 ($k = 6.0 \pm 0.1 \times 10^{-5}\ s^{-1}$, 3 measurements). A similarly slower decay in Krebs solution has been noted previously with the aziridinium ion from PrBCM (Young, Hiley & Burgen, 1972).

It should be noted that measurement of the yield of aziridinium ion is not a satisfactory test of the purity of acetylcholine mustard. In one preparation a second product, b.p. 40-41° C/0.2 mm, was isolated and identified as mustine, bis(2-chloroethyl)methylamine, by comparison of its i.r. and n.m.r. spectra with those of an authentic sample prepared by the method of Hanby & Rydon (1947). N.m.r. assignments (in $CDCl_3$, line positions in parts/10^6 downfield from tetramethylsilane): N-Me, 2.38, s; N-CH_2 methylenes, 4 protons, 2.82, t (J 6.7 Hz) and Cl-CH_2 methylenes, 4 protons, 3.57, t (J 6.7 Hz). The piperazinium dimers from both samples were likewise identical. Thus samples of acetylcholine mustard contaminated with mustine give a misleadingly high value of aziridinium ion (as measured by reaction with thiosulphate ion). As a rapid and reliable check of the purity of acetylcholine mustard samples, we have routinely used the relative integration values of the peaks in the n.m.r. spectrum (calculated from the number of protons involved in each resonance). In samples contaminated with mustine while there is almost no change in the line positions in the spectrum, there is a clear discrepancy from the theoretical integration.

Acetylcholine mustard was stored as the pure liquid at -10° C. Under these conditions solid material (presumably the piperazinium dimer) was slowly deposited and was filtered off at intervals, but the purity of the supernatant liquid remained good, even after storage for a year.

[^{3}H]-propylbenzilylcholine mustard

[^{3}H]-PrBCM (specific radioactivity 1.8 Ci/mmol and 1.4 Ci/mmol) (Burgen *et al.*, 1974a) was cyclized as a 0.13 mM solution in 10 mM phosphate buffer, pH 7.5, at room temperature (approx. 23° C) for 1 h, as described previously (Burgen *et al.*, 1974a). Under these conditions the yield of the aziridinium ion was 89%.

Irreversible binding of acetylcholine mustard

Longitudinal muscle strips from guinea-pig small intestine were prepared essentially as described by Rang (1964) and suspended in Krebs-Henseleit solution gassed with 5% CO_2 in O_2 at 30° C. After 1 h physostigmine (10^{-6} M) was added and the preincubation continued for a further 30 minutes. At the end of this period the strips were transferred to fresh Krebs solution containing physostigmine 10^{-6} M and freshly cyclized acetylcholine mustard and incubated for a given time on a shaking water bath. Control strips were similarly treated except that the Krebs solution contained no acetylcholine mustard. At the end of the incubation all strips were transferred to fresh Krebs solution and washed, usually for 30-40 min, before treatment with [^{3}H]-PrBCM 2.4 nM for 10 minutes. The reaction was terminated and non-covalently bound tritium removed by washing for 75 min with several changes of Krebs solution. The strips were then blotted dry, transferred to scintillation vials, weighed, treated with 0.7 ml Soluene (Packard) and allowed to stand overnight at room temperature, by which time the strips had dissolved completely. Ethoxyethanol (1 ml) and 10 ml butyl-PBD in toluene (0.4%, w/v) were added and the tritium determined by liquid scintillation counting.

Determination of the binding affinity of acetylcholine mustard

The irreversible binding of benzilylcholine mustard to muscarinic receptors in the longitudinal muscle of guinea-pig ileum can be described in terms of a scheme where the reversible drug-receptor complex (AR) once formed is

$$A + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AR \overset{k_2}{\rightarrow} AR'$$

converted into the covalently-bound complex (AR′) faster than it dissociates, i.e. $k_2 \gg k_{-1}$ (Gill & Rang, 1966). For acetylcholine mustard, an agonist whose action is terminated rapidly on washing and which gives a contractile response at a concentration and for an exposure time which produces no apparent covalent binding, it is highly probable that $k_{-1} \gg k_2$. This is the usual situation for active-site-directed irreversible inhibitors of enzymes. Thus for acetylcholine mustard the free-receptor fraction should initially decline exponentially with time, the apparent rate constant, k_{app} being related to the concentration of acetylcholine mustard [A], by the expression (Kitz & Wilson, 1962):

$$\frac{1}{k_{app}} = \frac{1}{k_2} + \frac{1}{k_2 . K_a} \cdot \frac{1}{[A]}$$

where $K_a = k_1/k_{-1}$, the apparent binding affinity of acetylcholine mustard.

The time course of [^{3}H]-PrBCM binding by muscarinic receptors in muscle strips approximates to an exponential curve (Burgen *et al.*, 1974a). Assuming that the rate constant for uptake of [^{3}H]-PrBCM is the same before and after treatment with acetylcholine mustard, then the proportion of the total uptake achieved after a given time will be the same in both cases. Thus the percentage uptake of the treated strips as compared to the control strips provides a measure of the free-receptor fraction.

Organ-bath studies

Muscle strips were suspended in a conventional 10 ml organ bath in Krebs-Henseleit solution at 30° C bubbled with 5% CO_2 in O_2. Contractions were recorded isotonically and washing was by overflow. Agonists were in contact with the tissue for 20-40 s, with a dose interval of 2 or 3 minutes.

For measurement of the equipotent molar ratio of acetylcholine mustard to acetylcholine (2 x 2 dose assay) 'nerve-free' muscle strips were prepared by the technique of Paton & Zar (1968). The advantage of these strips is that the contraction produced by physostigmine, which was present at a concentration of 10^{-6} M, is very much diminished (Cox & Lomas, 1971). The considerably larger amount of tissue required for the binding experiments made the use of 'nerve-free' strips there impracticable.

In experiments where the effect of extended incubation with acetylcholine mustard on the contractile response to carbachol was investigated, the usual procedure was first to establish a dose-response curve to carbachol and then to expose the tissue to the same dose of acetylcholine and for the same time as was to be tested for the mustard. After exposure to acetylcholine the tissue was allowed to recover from desensitization until a stable dose-response curve to carbachol could be obtained and the cycle then repeated, but with freshly cyclized acetylcholine mustard instead of acetylcholine. Recovery from desensitization caused by acetylcholine was complete by 60-90 min, although in some experiments this treatment resulted in a small parallel shift to the right of the log dose-response curve for carbachol and in some cases there was a small decrease in the resting length of the muscle.

Results

Potency of acetylcholine mustard as a muscarinic agonist

Acetylcholine mustard had an equimolar potency ratio of 4.3 (95% confidence limits 3.8-4.8) compared with acetylcholine in producing a contractile response in 'nerve-free' muscle strips in the presence of physostigmine 10^{-6} M. The presence of physostigmine produced an approximately 8-fold increase in the sensitivity of the strips to acetylcholine, but in two experiments in which the potency ratio was measured on normal muscle strips in the absence of physostigmine the values were 3.1 and 3.9, indicating that the two drugs must be similarly sensitive to cholinesterase. This is consistent with the report that the mustard is a substrate for bovine erythrocyte acetylcholinesterase (Hirst & Jackson, 1972). Hexamethonium (5×10^{-6} M) had no effect on the sensitivity to either agonist and the piperazinium dimer (10^{-4} M), a by-product of the cyclization reaction (Hanby & Rydon, 1947), was similarly without effect. The potency ratio is similar to that reported for intact intestinal segments from the guinea-pig, 6.1 ± 0.1 (Hirst & Jackson, 1972) and the rat, approx. 5 (Hudgins & Stubbins, 1972), both measured in the absence of a cholinesterase inhibitor.

Irreversible binding of acetylcholine mustard

Exposure of muscle strips to acetylcholine mustard in the presence of physostigmine (10^{-6} M) for increasing times led to a progressive inhibition of the subsequent binding of [^{3}H]-PrBCM compared with control strips which had not been treated with acetylcholine mustard. Approximately 28% of the uptake could not be inhibited, even after prolonged incubation with acetylcholine mustard (Figure 2) and this is taken to represent the non-specific binding of [^{3}H]-PrBCM. This percentage is, however, larger than the percentage of [^{3}H]-PrBCM binding (about 15%) which cannot be inhibited by reversible muscarinic agonists or antagonists added at the same time as the [^{3}H]-PrBCM, e.g. acetylcholine (Figure 3), during a 10 min incubation period.

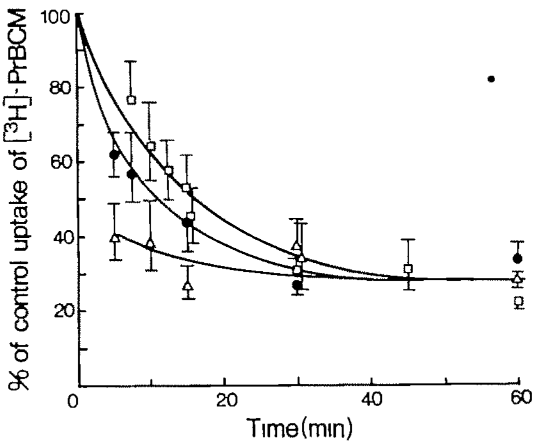

Figure 2 Progressive inhibition by acetylcholine mustard of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM) binding by muscle strips The experimental conditions are described in the methods section. The time is the time of incubation with acetylcholine mustard. Each point is the ratio of the uptake of [^{3}H]-PrBCM by 5-7 strips previously exposed to acetylcholine mustard to the amount bound by 5-7 untreated strips from the same animal. The vertical bars are the approximate 69% confidence limits. Acetylcholine mustard concentrations (M)· (□) 2.5×10^{-5}, (●) 4.9×10^{-5} and (△) 2.9×10^{-4}.

Recovery from block by acetylcholine mustard

The recovery from block by acetylcholine mustard was very slow. Muscle strips from three animals were exposed to acetylcholine mustard 2.5×10^{-5} M for 60 min at 30°C and then washed for periods ranging from 30 s to 2 h before labelling with [^{3}H]-PrBCM. To accentuate any recovery the washing after acetylcholine mustard was carried out at 37°C. No significant change in the level of the inhibition with time of washing was observed (Figure 4). If the difference between the 5 min and mean 120 min values were presumed to be real and assuming recovery follows first order kinetics, this would give a value for the

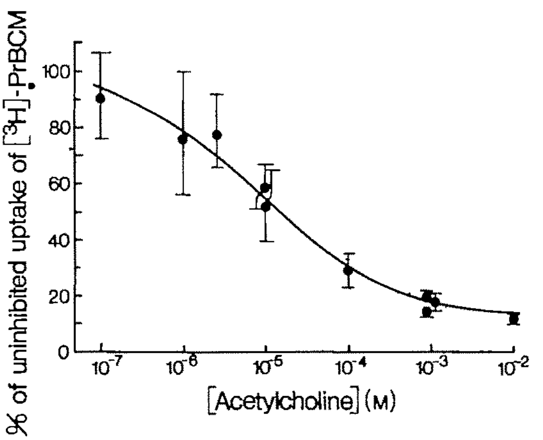

Figure 3 Inhibition of $[^3H]$-propylbenzilylcholine mustard ($[^3H]$-PrBCM) binding by acetylcholine. Neostigmine (10^{-7} M) or physostigmine (10^{-6} M) (for 2 points at 10^{-3} M and the point at 10^{-2} M acetylcholine) were added 30 min before, and acetylcholine 1 min before, $[^3H]$-PrBCM 2.4×10^{-9} M. Incubation was for 10 min at 30°C. Each point is the percentage of the uptake of $[^3H]$-PrBCM in the absence of acetylcholine (4-7 strips in each group). The vertical bars are the approximate 69% confidence limits.

rate constant of $3 \times 10^{-5}\ s^{-1}$ and it may well be that the true value for acetylcholine mustard is not greatly different from that for $[^3H]$-PrBCM at 37° C, $6 \times 10^{-6}\ s^{-1}$ (Burgen *et al.*, 1974a).

Rate of onset of block and the apparent binding affinity of acetylcholine mustard

The rate of decline of the free-receptor fraction with time for various concentrations of acetylcholine mustard is shown in Figure 5. The free-receptor fraction has been calculated from the receptor-specific uptake of $[^3H]$-PrBCM only, taking the non-specific uptake to be that portion, 28% (Figure 2), insensitive to acetylcholine mustard. The errors inherent in the measurements make a rigorous test of the predicted exponential relationship difficult, but the data for the three highest concentrations of the mustard suggest that it is obeyed, even though two of the points deviate markedly from the best straight line (fitted by inspection).

The rate constants (k_{app}) for each acetylcholine mustard concentration, obtained from the lines in Figure 5, are replotted in Figure 6 as $1/k_{app}$ against 1/[acetylcholine mustard]. The slope and intercept of this plot (cf. methods section) give a value for k_2 of $1.6 \times 10^{-3}\ s^{-1}$ (95% confidence limits. $1.1\text{-}3.0 \times 10^{-3}$) and for K_a $1.2 \times 10^5\ M^{-1}$ ($0.6\text{-}1.7 \times 10^5$).

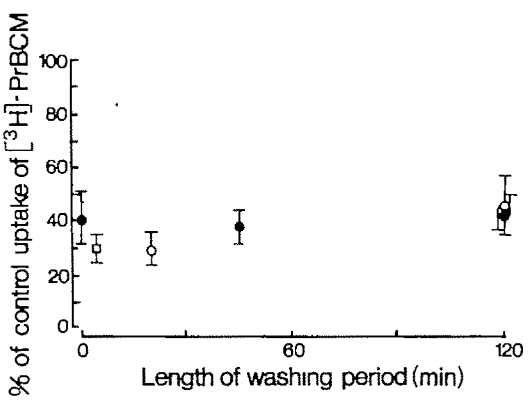

Figure 4 Rate of recovery from block by acetylcholine mustard. effect of varying the washing period at 37° C after exposure to acetylcholine mustard on the subsequent uptake of $[^3H]$-propylbenzilylcholine mustard ($[^3H]$-PrBCM). Each point is the percentage binding of $[^3H]$-PrBCM by 4-5 muscle strips exposed to acetylcholine mustard 2.5×10^{-5} M for 60 min compared with the binding by 4-5 untreated strips from the same animals. The vertical bars are the approximate 69% confidence limits. The different symbols denote different animals.

Effect of acetylcholine mustard on the contractile response to carbachol

The irreversible binding of acetylcholine mustard to muscarinic receptors demonstrated by the inhibition of the subsequent binding of $[^3H]$-PrBCM is in marked contrast with the report (Hirst & Jackson, 1972) that exposure of segments of guinea-pig ileum to acetylcholine mustard 10^{-4} M for 1 h (but in the absence of a cholinesterase inhibitor) produced no irreversible antagonism of the contractile response to acetylcholine or pilocarpine. In view of this discrepancy a number of organ bath experiments were carried out in which the effect of exposure of the longitudinal muscle strips to acetylcholine mustard (9.8×10^{-6} M for 20 min, 2.5×10^{-5} M for 30 min and 4.9×10^{-5} M for 30 min) on the subsequent response to carbachol was compared with exposure to the same dose of acetylcholine. Physostigmine (10^{-6} M) was added approximately 3 min before the acetylcholine or acetylcholine mustard, but was washed out at the end of the incubation and was not present during measurement of the response of the tissue to carbachol (cf. methods section).

The effect of exposure to acetylcholine

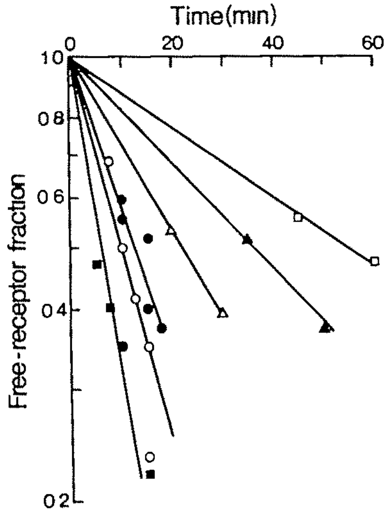

Figure 5 The rate of decline of the free-receptor fraction as a function of acetylcholine mustard concentration. The derivation of the free-receptor fraction and the experimental conditions are described in the methods section. In calculating the free-receptor fraction the non-specific component of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM) uptake was taken as 28%. The error bars have been omitted for clarity, but they were generally comparable with those of a similar level of inhibition in Figure 2. The data represent measurements on a total of 261 muscle strips. Acetylcholine mustard concentrations (M): (□) 1.23 x 10^{-6}; (▲) 2.2 x 10^{-6}; (△) 4.9 x 10^{-6}; (●) 9.8 x 10^{-6}; (○) 2.5 x 10^{-5} and (■) 4.9 x 10^{-5}.

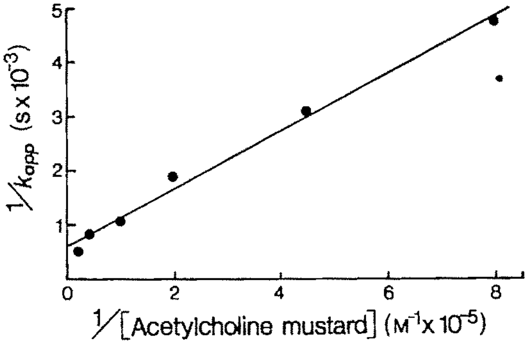

Figure 6 Plot of $1/k_{app}$ against 1/[acetylcholine mustard]. The values of k_{app} were derived from the slopes of the lines in Figure 5. The line is a least mean squares regression line.

mustard varied, depending on the dose employed, although the time course of the fade from the contraction produced was very similar to that after the same dose of acetylcholine. After the lowest dose (9.8 x 10^{-6} M for 20 min) there was a decrease in the resting length (to approx. 50% of previous) which was not influenced either by washing or by atropine (10^{-5} M). Carbachol produced small contractions superimposed on the raised baseline. In one experiment there was also an increase in the spontaneous activity. This became more marked where the treatment with acetylcholine mustard was 2.5 x 10^{-5} M for 30 minutes. Washing at 1 min intervals minimized the fluctuations without reducing the raised baseline (approx. 55% of previous maximum contraction), but with 3 min intervals the periodicity of the mechanical stimulus caused by washing produced slow oscillations of relaxation and recovery to the raised baseline. Carbachol still produced a response and indeed in one experiment this seemed to be much greater than would have been anticipated from the binding experiments, but the erratic baseline and the spontaneous slow oscillations made quantitative interpretation impossible. The spontaneous activity and raised baseline remained to some extent in two experiments where acetylcholine mustard 4.9 x 10^{-5} M for 30 min was employed, but in two other experiments after this dose of the mustard there was no change in the baseline and the response to carbachol was practically abolished.

The contrast between these observations and those of Hirst & Jackson (1972) is readily explained by the presence of physostigmine in the present experiments. This is supported by an experiment in which two muscle strips from adjacent segments of the intestine were set up in separate organ baths and both exposed to acetylcholine mustard 4.9 x 10^{-5} M for 30 min, one with and one without physostigmine 10^{-6} M. With physostigmine present the response to carbachol was practically abolished, but in its absence the result was similar to that reported by Hirst & Jackson (1972). After an initial instability the baseline returned to its original level and carbachol produced contractions not much smaller than the control responses, although the log dose-response curve was somewhat flattened.

Discussion

The inhibition of the binding of [^{3}H]-PrBCM to longitudinal muscle strips by acetylcholine mustard leaves little doubt that acetylcholine mustard does bind irreversibly to muscarinic receptors though its complex effects on the contractile properties of the tissue indicate that at the concentrations necessary for the inhibition of uptake it most probably has multiple actions on the tissue. This is not surprising, since the three-membered aziridinium ring is a powerful electrophile and there is likely to be a wide choice of nucleophilic reaction partners, such as thiol or amino groups, present in the tissue. The lack of specificity of other aziridinium ion-forming drugs, e.g. dibenamine, when used at concentrations above 10^{-6} M is well known and at 10^{-5} to 5×10^{-5} M, as required for acetylcholine mustard, the binding affinity for secondary sites need not be high if covalent bond formation is efficient, i.e. if k_2 is reasonably large. Thus although it is tempting to suppose that the increased tone which follows exposure to acetylcholine mustard might represent a permanent activation of the receptor, such as Silman & Karlin (1969) have observed for bromoacetylcholine bound to the electroplax of *Electrophorus electricus*, it seems more likely that this is the result of some other action of the mustard. In particular while there is no evidence that the electroplax shows the phenomenon of desensitization (Higman, Podleski & Bartels, 1963; Weber & Changeux, 1974), in intestinal smooth muscle permanent activation would be expected to lead to receptor desensitization, effectively producing irreversible antagonism. It may also be noted that whereas permanent activation of the electroplax by bromoacetylcholine is sensitive to (+)-tubocurarine (Silman & Karlin, 1969), the decreased resting length of the muscle strips after acetylcholine mustard was not influenced by atropine.

The use of the inhibition of [^{3}H]-PrBCM binding as a criterion for irreversible binding to muscarinic receptors circumvents the difficulties of multiple sites of action of acetylcholine mustard but it does, of course, demand that the binding of [^{3}H]-PrBCM which can be inhibited by acetylcholine mustard should represent muscarinic receptor binding. Evidence has been presented elsewhere that the uptake of [^{3}H]-PrBCM sensitive to atropine and other muscarinic drugs in intestinal muscle strips (Burgen *et al.*, 1974a), mammalian cerebral cortex (Burgen, Hiley & Young, 1974b) and chick amnion (Cuthbert & Young, 1973) is receptor-specific. However, there is a discrepancy between the extent of the maximum inhibition of [^{3}H]-PrBCM uptake by acetylcholine mustard (approximately 70%) and that by atropine or acetylcholine (approx. 85%). The reason for this difference is not clear and at present there is little evidence to support an explanation that the discrepancy reveals two populations of receptors in the proportion 70:15, both of which bind acetylcholine, but one of which does not react readily with acetylcholine mustard. It is still an open question whether all the receptor sites bound by [^{3}H]-PrBCM are involved in mediating the contractile response, but there is no good evidence to suggest that the 15% could represent such a fraction with which acetylcholine mustard forms no covalent bond. The behaviour of the tissue after large doses of acetylcholine mustard compromises any quantitative comparison of the contractile response before and after treatment and it has been shown that the discrepancy with previously published work (Hirst & Jackson, 1972) can be ascribed to the absence of a cholinesterase inhibitor. Similarly it seems likely that Hudgins & Stubbins (1972) would have observed a greater effect on the rat jejunum had they protected the mustard against enzymatic hydrolysis.

Taking the figure of 28% of the uptake of [^{3}H]-PrBCM to represent the effective non-specific binding, it has been shown that from an analysis of the decline of the free-receptor fraction values can be obtained for the rate constant of the alkylation reaction, k_2, and for the apparent binding affinity of acetylcholine mustard, which appears to correspond well with the concentration of acetylcholine required (approx. 10^{-5} M) for half-maximal inhibition of [^{3}H]-PrBCM binding. However, there are difficulties in interpreting these values. The prime difficulty is the lack of knowledge of receptor mechanisms, a problem which has recently been discussed by Rang (1973). Interpretation is further complicated by the fact that binding studies with agonists require the agonist to be present in relatively high concentrations for appreciable periods of time, conditions which favour receptor desensitization. It has recently been demonstrated (Young, 1974) that the pre-exposure of longitudinal muscle strips to a high concentration of carbachol followed by a brief wash changes both the position and the slope of the curve relating inhibition of [^{3}H]-PrBCM binding to carbachol concentration. Thus ongoing desensitization during exposure to the agonist may be the reason why binding curves for agonists obtained from the competitive inhibition of [^{3}H]-PrBCM binding have Hill coefficients of approx. 0.4-0.5 (Burgen & Hiley, 1974; Young, 1974), instead of the value of unity expected from a simple mass-action equilibrium. The curve for acetylcholine (Figure 3) has a Hill coefficient of 0.54. It is thus of particular interest that the kinetics of the irreversible binding of acetylcholine mustard appear to fit well with a scheme involving a simple drug-receptor equilibrium as the first step.

The reason for this contrast is uncertain. It is, however, notable that predesensitization of muscle strips results in an increase in the Hill coefficient for carbachol binding from 0.4 to 0.9 (Young, 1974), i.e. after desensitization the binding curve approximates to the theoretical curve for a mass-action equilibrium The concentrations of acetylcholine mustard employed and the period of incubation with the tissue are such that it is very likely that at least some desensitization will have occurred and it is possible that it is extensive If this is the case then the binding affinity, K_a, derived for acetylcholine mustard, 1.2×10^5 M^{-1}, may well be a measure of the affinity for the desensitized receptor

In interpreting K_a it must in any event be borne in mind that the observation that a plot of $1/k_{app}$ against 1/[acetylcholine mustard] is linear does not provide proof that the simple reaction scheme adopted (cf methods section) is correct, since the introduction of further reversible complexes between AR and AR$'$ does not alter the linear relationship, provided that the alkylation reaction is still the slowest step, although the constants become combinations of the various rate constants. Consequently K_a must be regarded as a kinetic constant whose meaning, just as with K_m in the normal Michaelis-Menton treatment of enzyme kinetics, will only become clear when the exact mechanism is known.

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EFFECT OF MORPHINE ON ADRENERGIC TRANSMISSION IN THE MOUSE VAS DEFERENS. ASSESSMENT OF AGONIST AND ANTAGONIST POTENCIES OF NARCOTIC ANALGESICS

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1 Morphine inhibits the electrically evoked (0.1-0.15 Hz, 1 ms) contractions of the longitudinal muscle of the mouse vas deferens but not of the rabbit, guinea-pig, rat, cat, hamster or gerbil. This effect is stereospecific and is antagonized by naloxone or naltrexone.

2 Normorphine is equiactive with morphine but its effects are more rapid in onset and decline.

3 In the mouse vas deferens, the resting outflow of tritium-labelled catecholamines is unaffected by morphine. The electrically evoked outflow is depressed by morphine or normorphine in a dose-dependent manner. The ID_{50} for inhibition of contraction and for depression of outflow is 0.5 μM.

4 The relative agonist potencies of compounds without antagonist component (codeine, pethidine, morphine, normorphine, heroin, levorphanol, Ba-20227, etorphine) show good correlation with the relative agonist potencies determined in the guinea-pig ileum and for analgesia in man.

5 For compounds with dual agonist and antagonist properties, the dose-response curves for agonist activity are shallow. When the lowest concentrations giving a depression of the contraction of the mouse vas deferens are used, a good correlation is obtained with the guinea-pig ileum.

6 The relative antagonist potencies of naloxone, nalorphine, levallorphan and cyclazocine agree well with those obtained in the guinea-pig ileum; these, in turn, correlate well with the values obtained in the morphine-dependent monkey.

7 The fact that the agonist effects of drugs with dual agonist and antagonist action show little or no dependence on concentration, makes the mouse vas deferens particularly suitable for the assay of antagonist activity.

8 As an assay preparation, the mouse vas deferens is less robust and consistent in its responses than the guinea-pig ileum.

Introduction

Henderson, Hughes & Kosterlitz (1972) have shown that contractions of the mouse vas deferens elicited by stimulation of the intramural nerves are inhibited by low concentrations of morphine. This effect was antagonized by naloxone and thus appeared to be mediated by specific morphine receptors. It was also shown that the inhibition of the contractions was associated with a depression of the evoked noradrenaline output as measured by bioassay. The aims of this paper are, first, to confirm and extend by radiochemical techniques the finding that noradrenaline output is depressed by morphine. Secondly, the mouse vas deferens was examined as a possible model for the prediction of the agonist and antagonist properties of narcotic analgesic drugs and the results compared with the findings obtained on the guinea-pig ileum (Kosterlitz & Watt, 1968; Kosterlitz, Lord & Watt, 1972; Kosterlitz, Waterfield & Berthoud, 1974). Some of the results have been described at a meeting of the British Pharmacological Society (Hughes, Kosterlitz & Leslie, 1974).

Methods

Experimental procedures

Albino mice of the T/O inbred strain, weighing 30-35 g, were killed by stunning or by exposing them to an atmosphere of CO_2. Both vasa deferentia were dissected out as a single unit connected by the tissue at the junction with the seminal vesicle. They were carefully stripped of adhering fat, connective tissue and blood vessels, and then gently pressed to expel their seminal contents.

For the investigation of the relationship between contraction and noradrenaline output and the effect of drugs on this relationship, five pairs of vasa were mounted in an organ bath of 3 ml (Hughes, 1972). When contractions only were examined, one pair of vasa was used. Longitudinal contractions were recorded by attaching the upper end of the preparation to a light spring connected to an isometric transducer. The tissues were bathed in modified Krebs solution containing ascorbic acid (0.1 mM) and sodium edetate (0.027 mM), gassed with 95% O_2 and 5% CO_2. A tension of 100-150 mg was applied to single preparations and 200-300 mg to multiple preparations. The intramural nerves were stimulated with rectilinear pulses (1 ms duration, supramaximal voltage) passing between a platinum point source at the bottom and a ring (10 mm wide) of platinum foil fixed to the top of the organ bath.

For the assay of agonist and antagonist potencies isotonic contractions were recorded by means of a photoelectric transducer. Only a single vas deferens was used and the tension was 100 mg. The organ bath and the electrode arrangements were similar to those already described but the Krebs solution did not contain ascorbic acid and sodium edetate.

Estimation of noradrenaline output

The tissues were incubated at 35° C, either for 45 min in Krebs solution containing (–)-[7-^{3}H]-noradrenaline (100 ng/ml, 40 μCi/μg = 6.8 Ci/mmol) or for 2 h in Krebs solution containing L-[3,5-^{3}H]-tyrosine (0.25 μg/ml, 221 μCi/μg = 40 Ci/mmol). After incubation, the tissues were rinsed for 30 min in fresh Krebs solution, changed every 5 minutes. They were then mounted in the organ bath as described above and washed for a further hour. In each experiment a resting sample of the total bathing fluid was collected for 4 min prior to a stimulation period of 4 min at 0.25 Hz. The catecholamines and their deaminated metabolites were isolated on short alumina columns (Boadle-Biber, Hughes & Roth, 1970); the columns were eluted with 1.5 ml of 0.15 M perchloric acid. The radioactivity was estimated in 0.5 ml of the eluate by liquid scintillation spectrometry. The results were corrected for a column recovery of 72%.

Assessment of agonist and antagonist potencies of narcotic analgesics

The method was similar to that used for the guinea-pig ileum (Kosterlitz & Watt, 1968; Kosterlitz *et al.*, 1974). The concentration of the drug which causes 50% inhibition of the evoked longitudinal contraction (ID_{50}) was used to characterize agonist activity and the dissociation or equilibrium constant (K_e) to characterize antagonist activity.

Determination of ID_{50} Values of ID_{50} were determined either from log dose-response curves or more often by the 'single dose' method. When the latter method was used, the preparation was set up and stimulated at 0.1 Hz for 20 min, the bath fluid being renewed several times. When the size of the contractions had become constant, two successive dose-response curves for normorphine were constructed, the exposure time to reach maximum effect being 60-90 s and the dose cycle 7 minutes. Fifteen minutes after the last dose of normorphine had been washed out, the compound to be tested was added. The dose varied according to its agonist activity; an amount was chosen to give a depression of the contraction of 20-40%.

If the slope of the dose-response curve of the compound under test was similar to that of normorphine, the value of ID_{50} was obtained by extrapolation. While in the guinea-pig ileum this method was used for agonists and for compounds with dual agonist and antagonist activities, this was not possible in the mouse vas deferens. As will be shown in the results section, the dose-response curves of the great majority of compounds with dual action is very shallow so that extrapolation is not permissible. To obtain a measure of the agonist potencies of these compounds, the lowest concentration of drug was found which gave a depression of about 20%. The concentration was then compared to the concentration of normorphine which gave the same depression and the potency ratio, nM normorphine/nM compound X, calculated.

Determination of K_e The value of K_e was obtained from $K_e = a/(DR-1)$, where a is the concentration (nM) of the compound to be tested and DR the dose-ratio, determined as follows (Figure 1). After the preparation had been exposed to the test compound as described for the 'single dose' method for the determination of ID_{50}, the concentration of normorphine (N_1) that would have caused the same depression was read off the mean of the second and third dose-response curves for normorphine obtained at the beginning and end of the experiment, respectively.

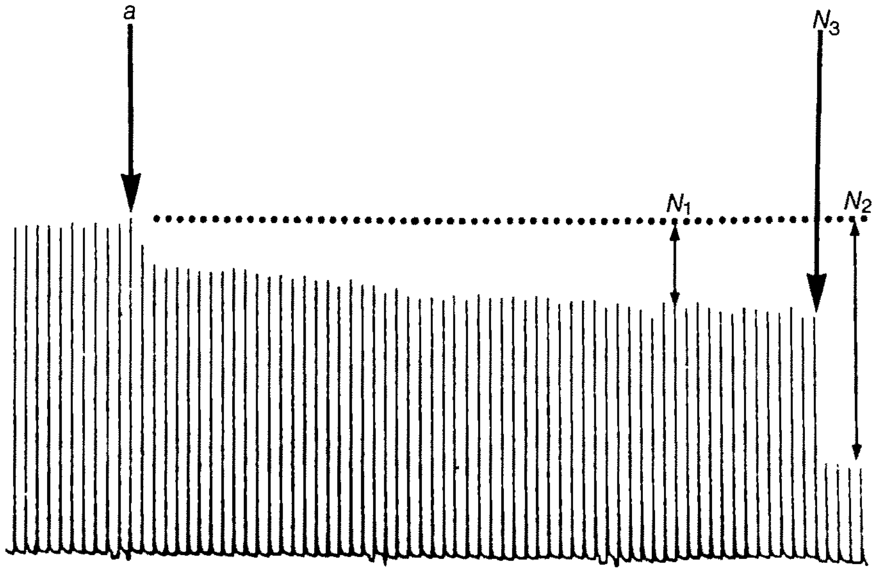

Figure 1 Measurement of the antagonist activity of a compound with dual agonist and antagonist actions, by determination of its equilibrium constant, K_e Mouse isolated vas deferens Isotonic recording of the longitudinal contractions induced by supramaximal electrical field stimulation at 0.1 Hz. The test drug was added at the arrow marked *a* and produced a depression of the twitch equal to a depression caused by normorphine in concentration N_1 After exposure of 10 min, normorphine was added to give a concentration of N_3, the total depression was equal to a reduction in the size of the twitch which would have been caused by normorphine in a concentration N_2 sr, in the absence of the test drug. $DR = N_3/(N_2-N_1)$, $K_e = a/(DR-1)$.

After the vas deferens had been exposed to the test drug for 5-10 min normorphine was added to the bath to give a concentration of N_3. The normorphine concentration (N_2) which would have a depressant effect equal to the combined actions of the test drug and the normorphine present in the bathing fluid was read off the mean dose-response curves for normorphine. The test drug was then washed out and normorphine added at intervals of 7 min until the sensitivity of the vas deferens to normorphine had returned to near its original level. If there was a change in sensitivity of more than 30%, the experiment was discarded. Finally, a third dose-response curve for normorphine was constructed. The values for N_1 and N_2 were then extrapolated from the mean of the second and third dose-response curves. The dose-ratio was then $DR = N_3/(N_2-N_1)$. N_3 should reduce the residual contraction by at least 20% and the total inhibition should not be more than 80%.

Solutions and drugs

The composition of the modified Krebs solution was (mM): NaCl 118, KCl 4.75, $CaCl_2$ 2.54, KH_2PO_4 0.93, $NaHCO_3$ 25, glucose 11.

The tritiated compounds, (−)-[7-^{3}H]-noradrenaline and L-[3,5-^{3}H]-tyrosine (Radiochemical Centre, Amersham) were purified on alumina columns before use.

The following drugs were used: adenosine triphosphate (Sigma); Ba-20227 (1-diethylaminoethyl-2-*p*-methoxybenzyl-5-nitro-benzimidazole hydrochloride, Ciba); codeine phosphate (B.P.); (−)-cyclazocine ((−)-α-2-cyclopropylmethyl-5,9-dimethyl-2′-hydroxy-6,7-benzomorphan, Sterling-Winthrop); (−)-cyclorphan hydrochloride ((−)-N-cyclopropylmethyl-3-hydroxy-morphinan, Dr M.D. Gates); dextrorphan tartrate (Roche Products); etorphine hydrochloride (Reckitt & Colman); dopamine hydrochloride (Koch-Light); heroin (diamorphine hydrochloride, B.P.); hexamethonium bromide (May & Baker); hyoscine hydrobromide (B.P.); levallorphan tartrate and levorphanol tartrate (Roche Products); lysergic acid diethylamide (Sandoz); morphine hydrochloride (B.P.); nalorphine hydrobromide (Burroughs Wellcome); naloxone hydrochloride and naltrexone hydrochloride (N-cyclopropylmethylnoroxymorphone) (Endo Laboratories); noradrenaline bitartrate (Winthrop); normorphine hydrochloride (Dr E.L. May); pethidine hydrochloride (Roche Products); phentolamine hydrochloride (Ciba); prostaglandin E_2 (Upjohn).

The stock solutions of the drugs were made in distilled water and that of cyclazocine in water plus the calculated amount of HCl. The concentrations are given as nM, μM or mM.

Results

Effects of narcotic analgesics on contractor responses to nerve stimulation

Morphine inhibited the contractions of the mouse vas deferens but no such action could be discerned in preparations from the rabbit, guinea-pig, rat, cat, hamster or gerbil. The effect was best seen when the mouse vas deferens was continuously stimulated at 0.1 or 0.15 Hz and the bath fluid was Krebs solution without magnesium ions. In Krebs solution containing 1.4 mM Mg^{++} the size of the contractions was only 40-60% of that in Mg^{++}-free solution; more importantly, the responses to morphine and other agonists were not consistent since the twitch tended to decline after repeated exposures to the drugs even when the tissues were washed vigorously between exposures. Dose-dependent inhibitions of the twitch were obtained with a series of narcotic analgesic agonists. Normorphine was used as reference because its action was rapid in onset and decline and it could be given at 5 min intervals without any apparent tachyphylaxis (Figure 2a,b). Morphine and the other agonists investigated had a slower onset and decline of action and the dose-cycle had to be lengthened to 15 min to avoid the development of tachyphylaxis. Naloxone (10-500 nM), which *in vivo* and *in vitro* is an antagonist without significant agonist activity (Blumberg & Dayton, 1974; Kosterlitz *et al.*, 1974), had no effect of its own on the twitch but reversed the inhibitory effects of the agonists (Figure 2b). Naltrexone, the N-cyclopropylmethyl analogue of naloxone, which has very weak agonist activity but as an antagonist is 3-4 times more potent than naloxone (Blumberg & Dayton, 1974; Kosterlitz *et al.*, 1974), completely antagonized the inhibitory effects of morphine and normorphine in the mouse vas deferens.

Table 1 shows the concentrations of various narcotic agonists required to depress the twitch by 50%; these ID_{50} values were calculated from dose-response curves obtained as shown in Figure 2. In each experiment at least two dose-response curves were obtained for the test drug, interspersed with dose-response curves for either morphine or normorphine. From the mean values for morphine and normorphine it follows that the mouse vas deferens is 6-7 times less sensitive than the guinea-pig ileum. The depressant effects of the narcotic agonists are stereospecific; it was found that the (+)-isomer of 3-hydroxy-*N*-methylmorphinan, dextrorphan, had only 0.1% of the potency of the (−)-isomer, levorphanol. The rank order of potency in the mouse vas deferens is in good agreement with that in the guinea-pig ileum; additional evidence in this respect will be given below.

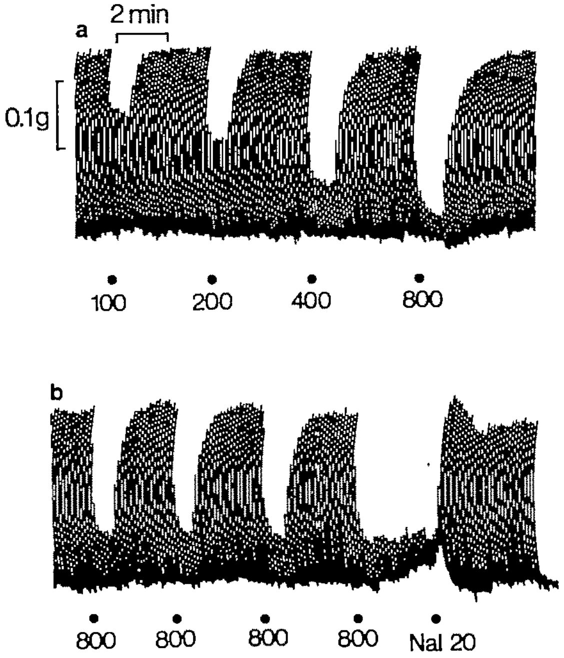

Figure 2 Mouse vas deferens. Effects of normorphine and naloxone on the longitudinal contractions evoked by electrical field stimulation (1 ms, 0.15 Hz, supramaximal voltage). (a) Effects of different concentration of normorphine (nM). (b) Absence of tachyphylaxis to normorphine (800 nM) and reversal by naloxone (Nal, 20 nM). Calibrations. tension, 50 mg; time, 2 minutes.

It should be noted that the strength of the electrical stimulation markedly influences the ID_{50} values of the narcotic agonists. When the current was reduced from a just supramaximal strength of 100 mA to 30 mA, the ID_{50} value for normorphine was reduced from 440 ± 42 nM to 120 ± 30 nM.

A number of compounds unrelated to narcotic analgesic drugs were investigated in order to confirm that the naloxone-induced reversal of the narcotic agonist action was indeed specific.

Adenosine triphosphate (ID_{50}, 150 mM), noradrenaline (ID_{50}, 10 μM), dopamine (ID_{50}, 3 μM), prostaglandin E_2 (ID_{50}, 10 μM), and lysergic acid diethylamide (ID_{50}, 0.14 μM) all inhibited the contractions elicited by electrical stimulation but these effects were not reversed by naloxone in concentrations (10-500 nM) that readily antagonized the inhibitory actions of the narcotic agonists.

Treatment of the vas deferens with phentolamine (30 μM) increased the twitch height by 30-80% and abolished the inhibitory actions of noradrenaline and lysergic acid diethylamide but did not reduce the effects of morphine or normorphine.

Effect of narcotic analgesics on noradrenaline output evoked by nerve stimulation

In order to exclude the possibility that ganglionic synapses might play a role in the action of morphine, the following experiments were carried out in the presence of hexamethonium (28 μM). It had previously been established that neither hexamethonium nor hyoscine (2.3-230 μM) affected the contractions elicited by nerve stimulation, nor did they modify the inhibitory effect of morphine on the twitch (Henderson *et al.*, 1972).

The resting outflow of ^{3}H-labelled catecholamines (after incubation with [^{3}H]-tyrosine, 1 expt, or with [^{3}H]-noradrenaline, 2 expts) was unaffected by morphine (1 μM). However, the stimulated output of labelled catecholamines was reversibly depressed by either morphine or normorphine. Figure 3 shows the marked inhibition of output from a vas deferens pre-incubated with [^{3}H]-tyrosine. The effect was readily reversed by washing the morphine out of the bath. When the tissue was treated with naloxone (50 nM) in the absence of morphine, there was no change in the stimulated output and a subsequent addition of morphine to the bath was without effect.

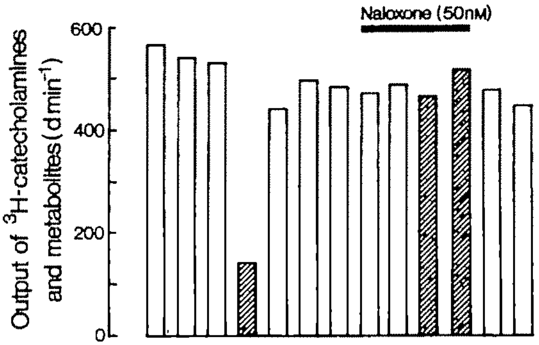

Figure 3 Effect of morphine and naloxone on the output of newly synthesized catecholamines from the mouse vas deferens The tissues were labelled with [^{3}H]-tyrosine as described in the methods section. Columns represent the output of ^{3}H-catecholamines and their deaminated metabolites after electrical stimulation at 0.25 Hz for 4 min every 15 min; the resting output of the preceding 4 min period has been deducted. Exposure to morphine (800 nM, shaded columns) reduced the output by 73%. Treatment with naloxone (50 nM, horizontal bar) did not affect the output of ^{3}H-catecholamines but the effect of morphine was abolished in the presence of the antagonist.

Table 1 The relative potencies of narcotic analgesic drugs in the mouse vas deferens and the guinea-pig ileum

Drug	*Mouse vas deferens ID_{50} (nM)*	*Relative agonist potencies (morphine or normorphine = 1)* Mouse vas deferens	Guinea-pig ileum
Ba-20227	4.3 ± 0.02 (4)	108	53
Levorphanol	168 ± 23 (5)	2.8	7.3
Heroin	350 ± 36 (4)	1.3	2.0
Normorphine	440 ± 42 (9)	1.0	1.0
Morphine	492 ± 53 (7)	1.0	1.0
Pethidine	16,000 ± 3,000 (5)	0.029	0.064
Codeine	55,000 ± 800 (5)	0.008	0.007

The ID_{50} values are the means ± s.e. mean obtained from dose-response curves; the number of observations are given in parentheses. The relative agonist potencies in the guinea-pig ileum are from Kosterlitz & Watt (1968) and Kosterlitz *et al.* (1974). The ID_{50} values for morphine and normorphine do not differ significantly; the combined means are 463 (n = 16) for the mouse vas deferens and 70.5 (n = 12) for the guinea-pig ileum, giving a ratio of 6.5.

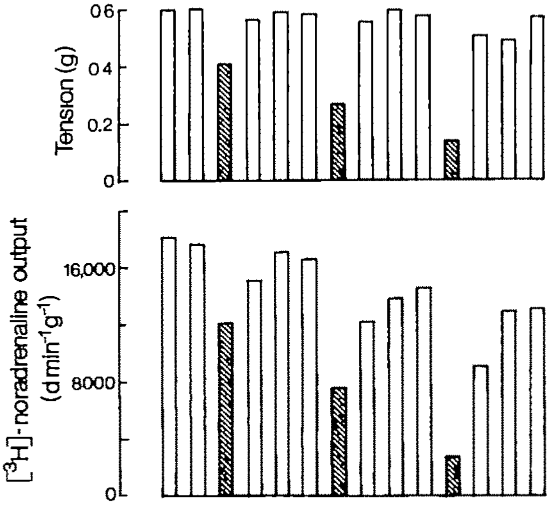

Figure 4 Effect of graded normorphine concentrations on the output of [^{3}H]-noradrenaline and contractions of the mouse vas deferens. The tissues were labelled with [^{3}H]-noradrenaline as described in the methods section. The Krebs solution contained phentolamine (30 μM) throughout the experiment. The upper panel shows the tension developed during stimulation (0.25 Hz for 3 min every 10 minutes). The lower panel shows the output of [^{3}H]-noradrenaline (including deaminated metabolites) during stimulation after deduction of the resting output for the preceding 3 minutes. Normorphine (shaded columns, 300, 600 and 1200 nM) caused a parallel reduction in both the output of [^{3}H]-noradrenaline and in the mechanical response.

The effects of morphine or normorphine on output were related to the dose. Figure 4 shows an experiment in which the vasa deferentia were first incubated with tritiated noradrenaline and then treated with phentolamine (3.1 μM), which increased the stimulated output threefold compared to outputs from untreated tissues and thus made possible the investigation of effects of a range of normorphine doses. Normorphine (300, 600 and 1200 nM) reduced the output by 35%, 60% and 80%, respectively, and the contractions by 32%, 53% and 76%. From four of these experiments it was calculated that the ID_{50} for inhibition of the contraction by normorphine was 510 ± 30 nM whilst the ID_{50} for inhibition of the output was 465 ± 58 nM. The difference between these values was not significant.

Assessment of agonist potency of compounds with dual agonist and antagonist actions

No difficulties were encountered in the assessment of the agonist potencies of compounds without antagonist component, either by dose-response curves (Table 1) or by the 'single dose' method.

As far as compounds with dual agonist and antagonist actions are concerned, their agonist potencies can be readily assessed in the guinea-pig ileum because the dose-response curve of their depressant action on the contractile responses has a slope near to that of agonists without antagonist component (Kosterlitz & Watt, 1968). Only when the agonist activity is small in comparison to the antagonist activity, is the dose response shallow and an estimate of the ID_{50} value becomes impossible (Kosterlitz *et al.*, 1972; 1974).

However, in the mouse vas deferens, all compounds which show antagonist activity have shallow dose-response curves for their agonist action. An example is given in Figure 5, showing dose-response curves for levorphanol and its N-allyl analogue, levallorphan. In an attempt to improve the slope, the interval between doses was increased from 7-40 min (Figure 5b) but without effect. It follows from these findings that the agonist potency of levallorphan relative to levorphanol was at its maximum when a low concentration was used. Therefore, with a compound with dual agonist and antagonist action, the lowest concentration was determined which gave a depression of about 20% of the evoked contraction. For the calculation of the relative agonist potency, the value obtained was related to the equiactive concentration of normorphine read from a dose-response curve. The values obtained agree within a factor of 1.5-3 with those found in the guinea-pig ileum (Table 2).

When the values of the relative agonist potencies of compounds with or without antagonist component were related to the relative potencies obtained in the guinea-pig ileum, the correlation coefficient was $r = 0.967$ ($n = 12$) and the regression line $y = 2.25x - 33.8$; the mean value of the ratios, relative potency in the vas deferens/relative potency in the ileum, did not differ significantly from unity (1.08 ± 0.21, $n = 12$). To assess the significance of the deviation of the slope from unity and of the constant from zero, it was necessary to establish whether or not the standard deviations of the potencies differed significantly over the wide range examined. Analysis showed that the standard deviations were closely correlated to the potencies ($r = 0.995$ for the ileum and $r = 0.997$ for the vas deferens). Since the ratios, standard deviation/potency, were not significantly correlated to the potencies ($r = 0.349$ for the ileum and $r = 0.142$ for the vas deferens), it was concluded that a logarithmic transformation would be suitable. This treatment would also give more equal weight to the widely

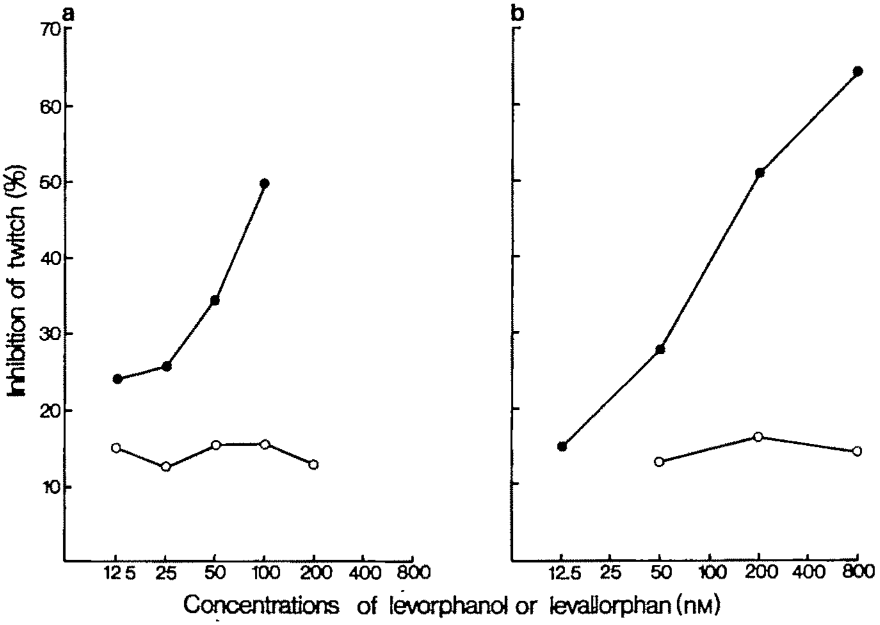

Figure 5 Dose-response curves for the inhibition of the evoked longitudinal contraction of the mouse vas deferens. Abscissae, concentration of levorphanol (●) or levallorphan (○); ordinates, inhibition of contraction (%). Dose-cycle 7 min in (a) and 40 min in (b).

varying potencies which had been obtained from log concentration-response curves.

After logarithmic transformation, the correlation coefficient for the relative potencies in the ileum and vas deferens was $r = 0.979$ which was similar to the value found for the non-transformed data. The slope of the regression line (Figure 6) was now close to unity (b = 1.02) and the constant near to zero (a = 0.07); these findings suggest that the values obtained in the two preparations vary in direct proportion to each other. When the relative potencies obtained in the mouse vas deferens, without codeine and Ba 20227, were compared to the relative potencies in human analgesia, r was 0.960 ($n = 10$) for the non-transformed data and 0.944 after logarithmic transformation. The slope of the regression line for the logarithmic data was, however, different from unity (b = 0.72, a = 0.01). This deviation was not surprising because any direct proportionality would have been modified by the effects of drug distribution, biotransformation and excretion. Similar values ($r = 0.926$,

Table 2 The relative agonist potencies of compounds with dual agonist and antagonist actions in the mouse vas deferens and the guinea-pig ileum

Drug	*Relative agonist potencies (morphine or normorphine = 1)*	
	Mouse vas deferens	*Guinea-pig ileum*
(−)-Cyclorphan	141 ± 11.1 (4)	240
(−)-Cyclazocine	45.4 ± 1.3 (3)	35
Levallorphan	8.6 ± 1.0 (2)	16
Nalorphine	0.92 ± 0.04 (3)	2.8

The relative agonist potencies are the means ± s.e. mean; the number of observations are given in parentheses. The relative agonist potencies in the guinea-pig ileum are from Kosterlitz & Watt (1968) and Kosterlitz *et al.* (1974).

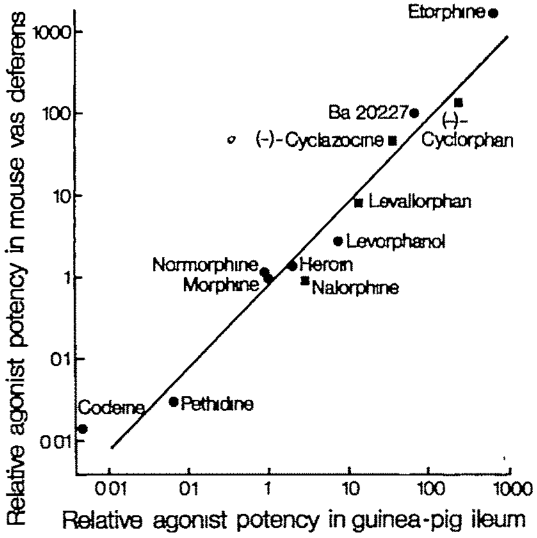

Figure 6 Correlation between the relative potencies of narcotic analgesics in the guinea-pig ileum and the mouse vas deferens. Abscissae, relative agonist potency in guinea-pig ileum (morphine = 1), ordinates, relative agonist potency in mouse vas deferens (morphine = 1). (●), Drugs with no significant antagonist action, (▪) drugs with dual agonist and antagonist actions. The values are plotted on a logarithmic scale. Correlation coefficient $r = 0.979$ ($n = 12$). The line has been drawn from $y = 1.02x - 0.07$. The values in the guinea-pig were obtained from Kosterlitz & Watt (1968), Kosterlitz *et al.* (1974) and Kosterlitz & Waterfield (1975). The values in the mouse vas deferens were determined by the 'single dose' method, with the exception of pethidine, levorphanol and Ba 20227 which were taken from Table 1.

b = 0.79, a = 0.03) have been found for the relationship between guinea-pig ileum and human analgesia (Kosterlitz & Waterfield, 1975).

Assessment of antagonist potency

The antagonist potency was determined by estimation of the dissociation or equilibrium constant K_e. The results obtained on four compounds are given in Table 3. Naloxone is an antagonist without significant agonist properties. Nalorphine, levallorphan and cyclazocine show agonist activities in both the mouse vas deferens (Table 2) and the guinea-pig ileum (Kosterlitz & Watt, 1968). Cyclorphan is a very potent agonist. Its antagonist properties cannot be demonstrated in the guinea-pig ileum; before a concentration is reached sufficient to produce a suitable dose-ratio, the contraction is completely inhibited by the agonist action (Kosterlitz *et al.*, 1974). In the mouse vas deferens, cyclorphan is also a potent agonist (Table 2) but because of its shallow dose-response curve, the antagonist properties were demonstrable (Table 3).

When the ratios of the K_e values obtained in the mouse vas deferens and the guinea-pig ileum were calculated, it was found that the mouse vas deferens was 3-6 times less sensitive to the antagonist action than the guinea-pig ileum. For reasons already discussed, no ratio was obtained for cyclorphan which is a potent antagonist in the mouse vas deferens.

Antagonist effects of mixtures of normorphine and nalorphine

Gyang & Kosterlitz (1966) drew attention to the fact that it was not possible to demonstrate the antagonist action of nalorphine in the guinea-pig ileum by adding mixtures of morphine and nalorphine to the organ bath.

Table 3 Comparison of the dissociation constants as measure of the antagonist potencies in the mouse vas deferens (K_{em}) and the guinea-pig ileum (K_{eg}).

Drug	K_{em} *(nM)*	K_{eg} *(nM)*	K_{eg}/K_{em}
Naloxone	3.66 ± 0.29 (4)	1.22 ± 0.03 (6)	0.33
Nalorphine	28.7 ± 3.2 (8)	4.47 ± 0.59 (6)	0.16
Levallorphan	7.11 ± 0.48 (6)	1.12 ± 0.23 (6)	0.16
(−)-Cyclazocine	4.86 ± 0.92 (7)	0.75 ± 0.19 (6)	0.15
(−)-Cyclorphan	1.58 ± 0 23 (6)	—	—

The values are the means ± s.e. mean; the number of observations are given in parentheses. The values in the guinea-pig ileum are from Kosterlitz & Watt (1968); Kosterlitz *et al.* (1972).

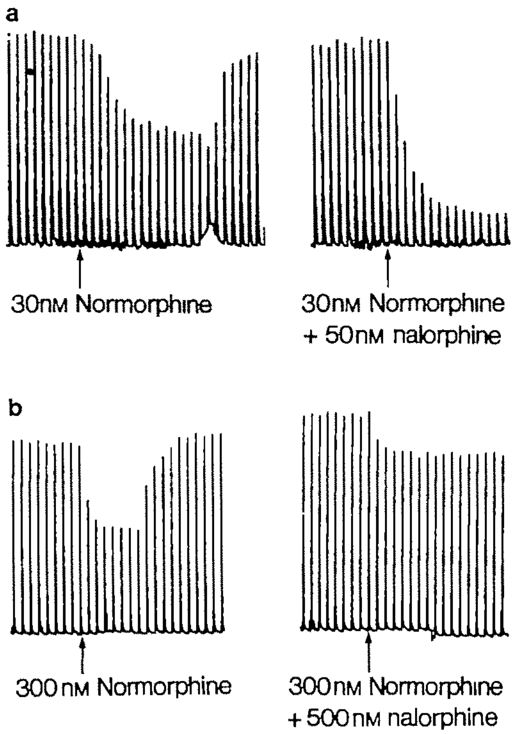

Figure 7 Comparison of the effects of mixtures of normorphine and nalorphine in (a) a segment of guinea-pig ileum and (b) in the mouse vas deferens. Stimulation was supramaximal at 0.1 Hz. The contractions of the ileum were recorded isometrically and those of the vas deferens isotonically

When such mixtures were added to the bath fluids in which a mouse vas deferens or a segment of ileum were suspended, quite different results were obtained with the two preparations (Figure 7). In the guinea-pig ileum, the agonist action of normorphine was increased by nalorphine, whereas in the mouse vas deferens the agonist action of normorphine was antagonized by nalorphine. This difference in effect was independent of the ratios of nalorphine to normorphine which varied from 0.2-33.

Discussion

The first aim of this investigation was the confirmation and further analysis of the finding of Henderson *et al.* (1972) that narcotic analgesics depress evoked noradrenaline output and the associated longitudinal contraction of the mouse vas deferens, and that this effect is due to an interaction with a morphine receptor.

It has been shown that these effects are stereospecific since they are produced only by levorphanol and not by its (+)-isomer, dextrorphan, and that they are antagonized by the antagonists naloxone and naltrexone. Thus, the requirements for a specific action have been fulfilled.

The classical view holds that noradrenaline released from sympathetic nerves in the vas deferens causes contractions of the smooth muscle by action on α-adrenoceptors. Ambache, Dunk, Verney & Zar (1972) have advanced reasons for doubting an excitatory transmitter role for noradrenaline in the vas deferens, but a number of authors have produced results consistent with the classical role of noradrenaline (Jacobowitz & Koelle, 1965, Wadsworth, 1973; Furness, 1974) and refute the hypothesis of Ambache and his co-workers. The most cogent reason for accepting the neurotransmitter role of noradrenaline in the vas deferens is that drugs that block noradrenaline release such as bretylium and cocaine (Hughes, 1972), lysergic acid diethylamide (Hughes, 1973) and morphine (Henderson *et al.*, 1972; this paper) cause a corresponding block of the contractile response to nerve stimulation. In the present investigation it was found that there was a very close correlation between the concentrations of normorphine required to produce similar inhibitions of the twitch and of the output of labelled catecholamine.

The possibility cannot be excluded that morphine may not directly inhibit noradrenaline release but block some more central link in the transmission process. Inhibition of ganglionic transmission cannot be entirely eliminated but it is unlikely since the effects of morphine are not reduced by hexamethonium or hyoscine (Henderson *et al.*, 1972), or by stripping the outer coat of the mouse vas deferens (Henderson & Hughes, unpublished observations).

The second aim of this paper was to examine whether the mouse vas deferens is a suitable model for the prediction of the agonist and antagonist properties of narcotic analgesic drugs for use in analgesia in man. There is little doubt that this preparation predicts with considerable accuracy the agonist activities of compounds without antagonist properties, e.g. pethidine, heroin and levorphanol. The potency of codeine is lower than would be expected from its analgesic properties; a similar low activity has been found in the guinea-pig ileum (Kosterlitz & Watt, 1968) and in binding studies on brain homogenates (Pert & Snyder, 1973). The discrepancy found with codeine is probably due to the fact that codeine undergoes biotransformation to morphine in man (Adler, 1963) but not in the mouse vas deferens, guinea-pig ileum or brain homogenate.

Complications arose with the assessment of agonist activities of compounds with dual agonist and antagonist properties since the dose-response curves of these compounds are very shallow. These difficulties were partly overcome by determining the lowest concentrations of these compounds which would depress the height of contraction by about 20% and relating this concentration to a concentration of normorphine causing the same depression. When this was done the correlation of the agonist activities with those found in the guinea-pig ileum was good. Since the results in the guinea-pig ileum predict satisfactorily the agonist effects in man (Kosterlitz *et al.*, 1974), the values obtained in the mouse vas deferens by the method of lowest active concentration also have good predictive properties.

The antagonist potencies of compounds with and without agonist properties are well correlated with those found in the guinea-pig ileum which in turn are in good agreement with those obtained in the morphine-dependent rhesus monkey (Kosterlitz *et al.*, 1974). The low sensitivity of the mouse vas deferens to the agonist action of higher concentrations of compounds with dual action makes this preparation particularly suitable for the estimation of the antagonist potency of these compounds.

It would appear therefore that the pharmacological properties of the morphine receptors in the mouse vas deferens, the myenteric plexus of the guinea-pig ileum, and the human central nervous system show considerable similarities, although the absolute sensitivity of the mouse vas deferens to agonists and antagonists is only 15-20% of that of the guinea-pig ileum. However, there are differences between the agonist activities of compounds with dual agonist and antagonist actions in the mouse vas deferens and guinea-pig ileum. This difference is mirrored by the variation in sensitivity of certain antinociceptive tests *in vivo*. Thus, compounds with dual agonist and antagonist actions have relatively little agonist activity in the mouse hot-plate test or the rat tail pressure test but show agonist activity correlated to human analgesia in the writhing test or the tail-shock test (Taber, 1974). Obviously, these differences are of importance for an understanding of the mechanisms underlying the action of narcotic analgesic drugs but on the evidence available at present any further discussion would be highly speculative.

A comparison of the usefulness of the two preparations, the mouse vas deferens and the guinea-pig ileum, for the prediction of the properties of new compounds indicates that the guinea-pig ileum is to be preferred because it is much more robust than the mouse vas deferens and gives constant twitch height over the long periods required for the assays. The agonist potencies of compounds with dual agonist and antagonist properties estimated by the guinea-pig ileum are more closely correlated to human values than those obtained by the mouse vas deferens unless special precautions are taken. On the other hand, the antagonist potencies of compounds with dual action are more readily assayed in the mouse vas deferens.

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THE OFFSET OF MORPHINE TOLERANCE IN RATS AND MICE

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1 In rats and mice made tolerant to morphine by pretreatment with the drug, the shift to the right of the log dose/analgesic response line for morphine from its position in naive animals occurs without significant change in slope provided that sufficient time is allowed for elimination of pretreatment drug

2 Responsiveness to the analgesic effects of morphine, given together with cycloheximide to prevent reinforcement of tolerance, was measured in rats (paw pressure method) and mice (hot plate method) at intervals during 1-23 days following cessation of a variety of regimens of tolerance-inducing drug treatments.

3 A biphasic pattern of recovery of responsiveness was observed, which was independent of the regimen or the drug (morphine, methadone or diamorphine) used to induce tolerance. Estimates of the rates of the first, fast phase are imprecise but the rate of the second phase of offset, from 4th day after cessation of pretreatment had, in rats, a mean half-time of 13.2 ± 0.53 days—for all pretreatments combined, there being no significant differences between the various pretreatment regimens employed. In mice, similarly, a biphasic recovery of analgesic responsiveness was seen after morphine pretreatment, the mean half-time of the slower phase being 17.4 days.

4 Precipitation of an acute withdrawal syndrome in rats by naloxone HCl given 6 h after the final injection of a tolerance-inducing treatment with morphine did not affect the subsequent rate of recovery from tolerance.

5 During the period following a tolerance-inducing pretreatment with morphine in mice, the rate of attenuation of the naloxone-evoked jumping response was faster than the rate of offset of tolerance.

Introduction

Opioid narcotic drugs produce numerous effects on neuronal function in the central nervous system, and it has proved difficult to identify separately those processes concerned in the mediation of particular opioid agonist actions or those involved in the genesis of tolerance and dependence. Identification of the mechanisms responsible for the phenomenon of tolerance may be assisted by knowledge of the rate of recovery from the tolerant state, because it seems likely that the rate of reversion towards the level of responsiveness to morphine seen in naive animals reflects the recovery from the underlying metabolic perturbation.

Reports on the rate of offset of opioid tolerance are sparse and conflicting; Cochin & Kornetsky (1964) report that in rats significant tolerance to morphine was retained for more than one year following single or multiple doses of morphine, whereas Goldstein & Sheehan (1969) estimated the time of recovery from levorphanol tolerance in mice to be 'essentially the same, within the limits of experimental error, as the rate of onset of tolerance,' (that is, in their experiments, a half-time of 16-48 hours).

We have, therefore, reassessed the rate of recovery from tolerance to the analgesic effects of morphine in both rats and mice. A difficulty in measuring the level of morphine tolerance arises from the fact that the test procedure necessarily involves the administration of an opioid analgesic which then reinforces the phenomenon being measured. This problem has been circumvented in the present experiments by the simultaneous administration of a protein synthesis inhibitor with the test dose of opioid drug, a procedure which prevents the further development of tolerance whilst not affecting previously established tolerance (Cox, Ginsburg & Osman, 1968; Cox & Osman, 1970). A preliminary account of this work has been presented to a meeting of the British Pharmacological Society (Cox, Ginsburg & Willis, 1973).

Methods

Induction of opioid drug tolerance

All drugs were dissolved in 0.9% w/v NaCl solution (saline), and were given s.c. to both rats and mice, except for methadone which was given i.p. because when given s.c. it causes, in some animals, skin lesions at the site of injection. Injections were given twice daily, at 09 h 00 min and 17 h 00 min; on the starting day the first injection was given at 17 h 00 min and on the concluding day the final injection was given at 09 h 00 min. Control animals were given twice daily injections of saline simulating the regimens of drug administration which were as indicated in Table 1.

Assessment of analgesic responsiveness

Analgesic responsiveness was tested in rats by a pressure method during continuous intravenous infusion of morphine HCl with cycloheximide via an exteriorized cannula inserted under ether anaesthesia in an external jugular vein at least 18 h before the beginning of the infusion (Cox, *et al.*, 1968). The flow rate of the infusion was 0.5 ml/h and the infusate (in saline) contained cycloheximide such that each rat received 200 μg kg^{-1} h^{-1}. In most experiments the rate of concomitant morphine HCl infusion was 5 mg/kg^{-1} h^{-1}.

Analgesia was assessed in rats by estimation, at intervals during an infusion, of responsiveness to a painful stimulus using an Analgesimeter (Ugo Basile, Milano) to measure the minimum load that must be applied to a rat's hind paw to cause the animal to react, see Randall & Selitto (1957).

In mice, analgesia was measured by a modification of the hot-plate method described by Woolfe & McDonald (1944); the surface temperature of the plate was 55° C. The response time to elicit the reaction (licking or blowing on the front paws, kicking the hind feet or hopping on all feet or jumping) was noted before and 30 min after a s.c. injection of morphine HCl (12 mg/kg) plus cycloheximide (20 mg/kg). Mice that did not react within 30 s were removed from the hot-plate to avoid damaging their paws. In fact, all mice responded within that time.

Assessment of morphine dependence in mice

The rate of spontaneous jumping or 'escape jumping' in mice is increased after an injection

Table 1 Pretreatments for induction of morphine tolerance in rats.

Day	1	2		3		4		5		6		7
Hour	17.00	09.00	17.00	09.00	17.00	09.00	17.00	09.00	17.00	09.00	17.00	09.00
Morphine A	10	10	20	20	20	20						
Morphine B	10	10	20	20	20	20	40	40	40	40	40	40
Morphine C	10	10	20	20	40	40	60	60	80	80	100	100
Morphine D	20	20	40	40	80	80	120	120	160	160	200	200
Methadone	10	10	10	10	12.5	12.5	12.5	12.5				
Diamorphine	5	5	10	10	15	15	20	20				

Doses refer to mg/kg of hydrochloride of each drug. Morphine and diamorphine were given s.c., methadone was given i.p.

with naloxone (10 mg/kg i.p.) following pretreatment with morphine and is often taken as a measure of dependence (Way, Loh & Shen, 1969; Cheney & Goldstein, 1971). The jumping rate was counted using the apparatus (Figure 8) described in the Appendix.

Results

Relationship of morphine dosage to analgesic response in naive and tolerant animals

Groups of 6 rats were given an intravenous infusion of morphine at 1,2,3,4 and 5 mg kg^{-1} h^{-1}. Cycloheximide was infused concurrently with the morphine HCl at a rate of 200 μg kg^{-1} h^{-1} to prevent the development of tolerance to the morphine during the course of infusion (Cox & Osman, 1970), and the nociceptive responsiveness for each rat was assessed by determination of the pressure threshold at 60 or 90 min intervals after the start of the infusion. At each infusion rate, the mean analgesic index rose during the first 2-3 h to reach a plateau level which was maintained to the end of the infusion (Figure 1). Similar experiments were carried out in groups of rats which had been rendered tolerant to the analgesic effects of morphine by pretreatment with the drug; these test infusions were administered 2 days or 7 days after cessation of pretreatment. The mean analgesic indices again reached a plateau after about 3 h of the infusion as with the naive animals but, naturally, greater amounts of morphine were required to produce analgesic effects of comparable magnitude. The steady state analgesic indices were plotted against the morphine infusion rates (on a logarithmic scale). The straight line fitting the points obtained in these experiments, with animals 7 days after cessation of the tolerance-inducing treatment, paralleled that for naive animals and was shifted to the right (Figure 2a). However, 2 days after the end of the pretreatment period the more marked shift to the right was accompanied by significant flattening of the log-dose response line.

Log-dose response curves were also determined in naive mice and mice 2 days after a period of 7 days treatment with morphine HCl (dosage schedule D, Table 1). Analgesic responsiveness was tested by the hot plate method. Single subcutaneous doses of morphine HCl were given and the analgesic response was measured 30 min later. There was no significant deviation from parallelism of the log-dose response curves for naive and tolerant animals (Figure 2b).

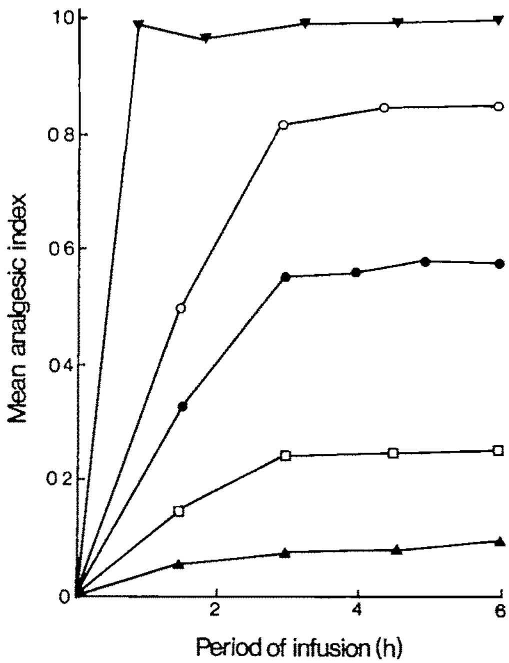

Figure 1 Time course of analgesic responses to different doses of morphine plus cycloheximide (200 μg kg^{-1} h^{-1}), infused intravenously for 6 h into conscious rats. Mean analgesic index was estimated from 4-6 rats. In this and subsequent figures s.e. mean are not shown where they are $<$ symbol signs. (▲) 1 mg kg^{-1} h^{-1} morphine HCl; (□)2 mg kg^{-1} h^{-1} morphine HCl; (●) 3 mg kg^{-1} h^{-1} morphine HCl, (○) 4 mg kg^{-1} h^{-1} morphine HCl; (▲) 5 mg kg^{-1} h^{-1} morphine HCl

Recovery of the analgesic response to morphine after the development of tolerance

Recovery following morphine pretreatment in rats. The offset of morphine tolerance was studied by measuring the analgesic response to a standard infusion of morphine, at various time intervals after cessation of the tolerance-inducing treatments. The choice of this approach, rather than the more expensive, arduous, and time consuming procedure of determining for each stage the dose of morphine that would elicit a standard analgesic effect, was justified in the light of the results described above, showing that the reduced responsiveness of tolerant animals could be described by a parallel shift to the right from the dose-response curve for morphine in naive animals.

On selected days after the termination of a period of morphine pretreatment, rats in separate groups of between 4 and 6 were given intravenous

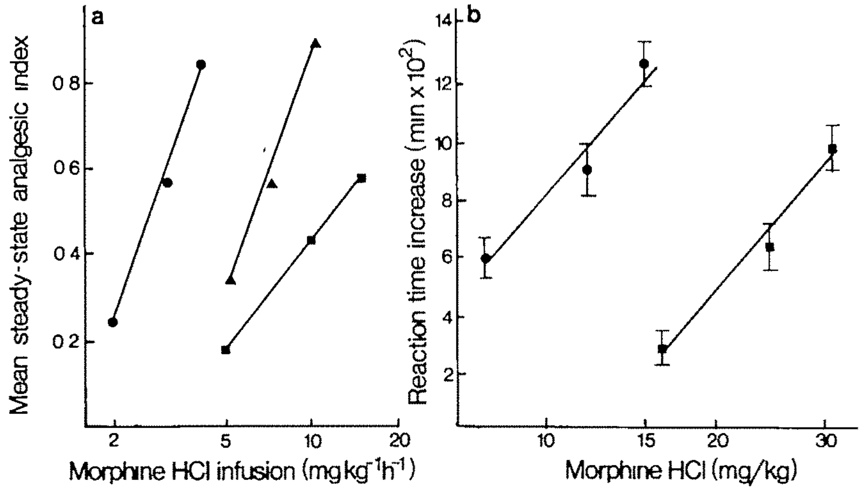

Figure 2 Log dose-response plots for morphine in naive and tolerant rats and mice. (a) In rats given morphine pretreatment C and tested for morphine responsiveness by a paw pressure method during continuous intravenous infusion of morphine plus a fixed dose of cycloheximide (200 μg kg^{-1} h^{-1}) (b) In mice given morphine pretreatment D and tested for morphine responsiveness by the hot-plate method, 30 min after s.c. injection of morphine HCl. (●) naive animals, (■) 2 days after cessation of pretreatment, (▲) 7 days after cessation of pretreatment

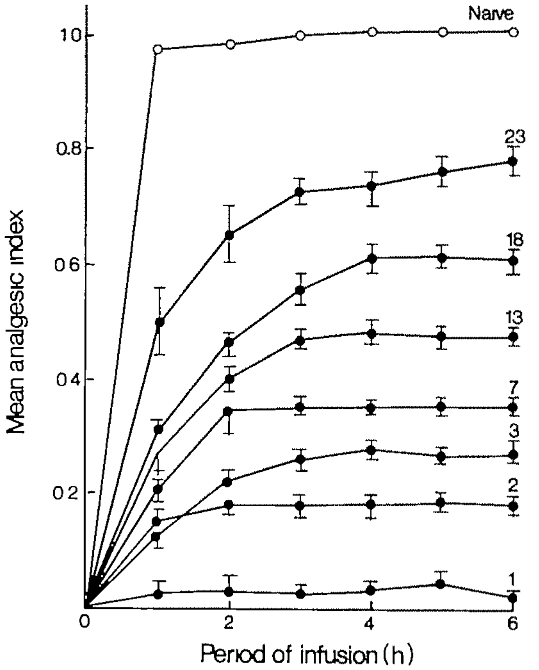

Figure 3 Antinociceptive responses during intravenous infusion of morphine HCl (5 mg/kg^{-1} h^{-1}) plus cycloheximide (200 μg/kg^{-1} h^{-1}) in rats during recovery from morphine tolerance (pretreatment C). Numbers on curves refer to the number of days after cessation of the pretreatment. Mean analgesic index was estimated from 4-6 rats Vertical bars indicate s.e. mean. (o) results from naive rats which received saline only in the pretreatment period.

infusions of morphine HCl (5 mg kg^{-1} h^{-1}) together with cycloheximide (200 μg kg^{-1} h^{-1}); pressure thresholds were determined at intervals during the infusion (Figure 3).

During each infusion the mean analgesic index value increased for the first 2-3 h when a steady state level was reached which was maintained for the rest of the infusion period. The steady state analgesic index increased progressively as the interval between the test infusion and the final dose of the pretreatment A plot of the steady state analgesic index value (on a logarithmic scale) against the days after termination of morphine pretreatment showed that the recovery of sensitivity to morphine occurred in two phases; an initial rapid phase of recovery followed by a slower phase (Figure 4a). Similar results were obtained for the three different regimens of morphine pretreatment, which were chosen to induce initial tolerances of varying intensity.

From the point of inflection on the curve (at about the fourth day) until offset of tolerance was virtually complete (up to 23 days) the points were well-fitted by straight lines, the slopes of which were similar for all tolerance-inducing treatments. The mean slopes during this phase correspond to a half-time for recovery of morphine sensitivity of 13.2 ± 0.53 days. No estimates of the rate of recovery during the initial rapid phase have been made because of low precision in the determination of the analgesic indices at the low values obtained in highly tolerant animals and because the testing procedure itself occupies a period of

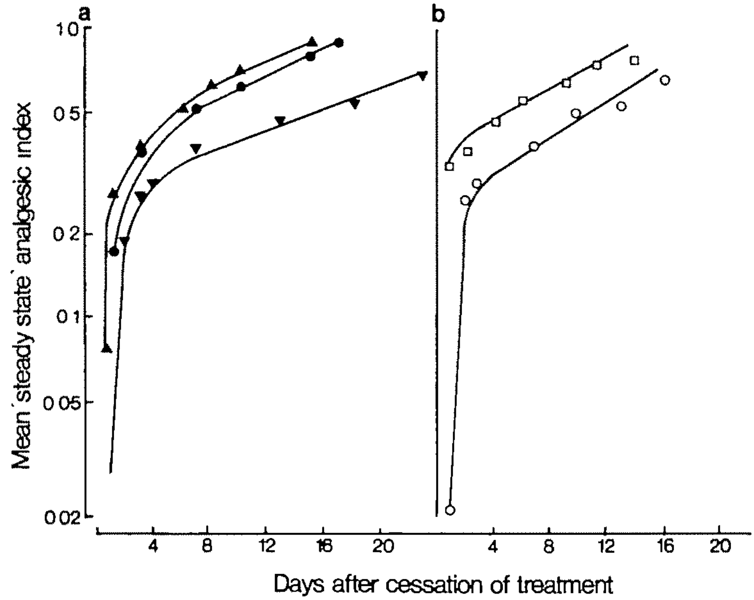

Figure 4 Rate of recovery of responsiveness to morphine HCl (5 mg/kg^{-1} h^{-1}) infused intravenously with cycloheximide (200 μg/kg^{-1} h^{-1}) in rats rendered tolerant to morphine by different pretreatments. Mean 'steady state, analgesic index was estimated from 4-6 rats. (a) (▲) morphine pretreatment A; (●) morphine pretreatment B, (▲) morphine pretreatment C; (b) (□) methadone pretreatment; (○) diamorphine pretreatment

time that is significant in relation to the recovery rate

Recovery following methadone or diamorphine pretreatment in rats. Measurements of the recovery of sensitivity to morphine after pretreatment of rats with methadone or diamorphine showed that the analgesic response returned in a similar biphasic manner following tolerance induction with these drugs (Figure 4b). From the fourth day onwards the points are fitted by straight lines whose slopes correspond to half-times for recovery of 14.2 and 11.4 days for methadone and diamorphine respectively.

Recovery following morphine pretreatment in mice At predetermined time intervals after the completion of the tolerance induction in mice, the response, to a s.c. injection of the standard dose of morphine HCl (12 mg/kg) plus cycloheximide (20 mg/kg), was tested by measuring the increase in reaction time on the hot plate; different groups of animals were used at each time of testing. When the responses were plotted on a logarithmic scale against the time after the final morphine dose, it was seen that once again recovery occurred in two phases. In mice the half-time for the second phase was estimated to be 17.4 days (Figure 5). The possibility that the protein synthesis inhibitor

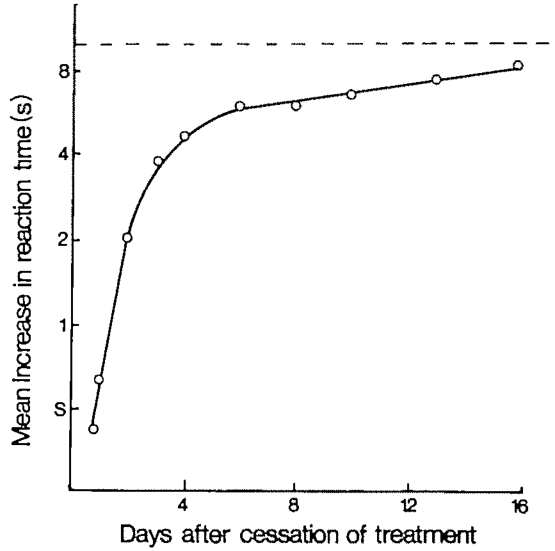

Figure 5 Rate of recovery of responsiveness to morphine in tolerant mice. Reaction time was estimated 30 min after a s.c. injection of morphine HCl (12 mg/kg) plus cycloheximide (20 mg/kg) in tolerant mice (observations in 16-25 animals). The horizontal broken line indicates the mean increase in reaction time in naive mice.

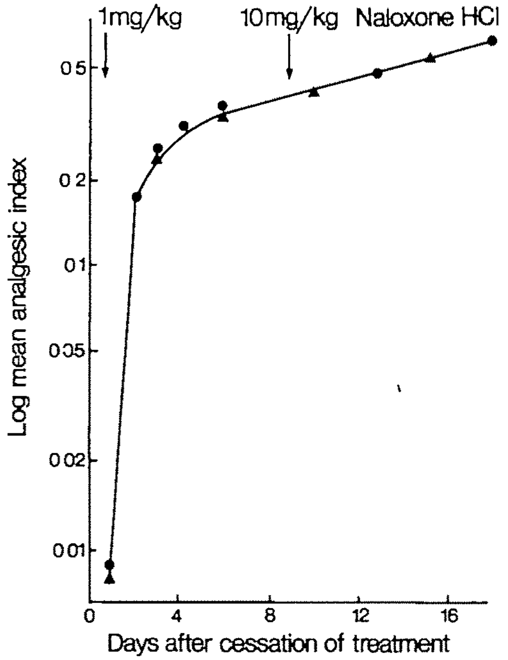

Figure 6 Lack of effect on recovery from morphine tolerance in rats of naloxone given 6 h and 9 days after the cessation of pretreatment C. Mean 'steady state' analgesic index was estimated from 4-6 animals. (▲) naloxone treated; (●) untreated with naloxone

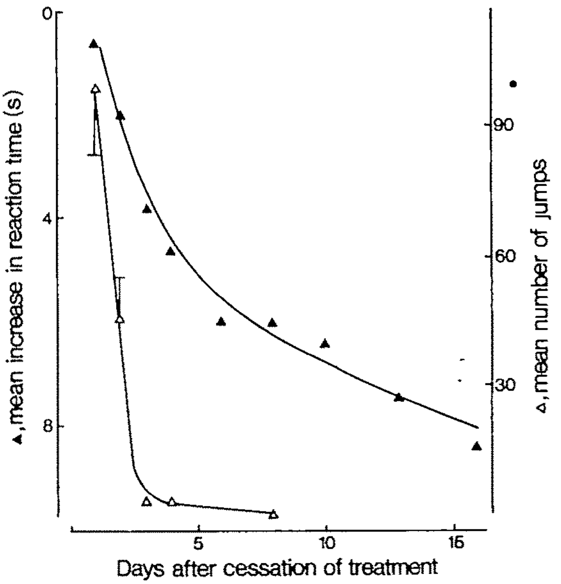

Figure 7 Comparison of offset of morphine tolerance and naloxone-elicited jumping in mice. Increase in hot plate reaction time was estimated 30 min after s.c injection of morphine HCl (12 mg/kg) plus cycloheximide (20 mg/kg). Mean number of jumps per mouse was determined during a 20 min period after i.p. injection of naloxone HCl (10 mg/kg; observations in 16-25 mice) The intersection of the abscissa and the left-hand ordinate gives the mean increase in hot plate reaction time in naive mice

alone might affect recovery was tested by giving one group of eight mice, cycloheximide (20 mg/kg s.c.) on the ninth day of recovery. The increase in reaction time produced by morphine and cycloheximide in these animals on the following day was not different from that in a control group of mice.

Effects of naloxone on recovery from morphine pretreatment in rats. It has been suggested that morphine antagonists such as nalorphine or naloxone interact competitively with morphine at a common receptor site (Cox & Weinstock, 1964; Grumbach & Chernov, 1965; Pert & Snyder, 1973) and that displacement of morphine from binding sites might be responsible for the rapid onset of withdrawal symptoms induced by these drugs in morphine-dependent animals (Goldstein, Aronow & Kalman, 1969). It was therefore of interest to investigate the effects of naloxone treatment on the recovery of sensitivity to morphine. A group of rats tolerant to morphine (pretreatment C, Table 1) received a dose of naloxone HCl (1 mg/kg s.c.) 6 h after the last morphine dose. This dose was sufficient to precipitate withdrawal symptoms namely diarrhoea, irritability, ptosis, headshakes, body trembling, etc., symptoms which were more intense and more acute than those observed following simple withdrawal of the drug. Different subgroups of these animals were then given the standard morphine and cycloheximide infusion on subsequent days and the steady state analgesic indices determined. Animals not tested for morphine responsiveness before day 9 of recovery received an additional dose of naloxone HCl (10 mg/kg s.c.); the second naloxone dose did not elicit any observable withdrawal symptoms.

The responses of the naloxone-treated rats were compared with those of a control group which received an identical morphine pretreatment but did not receive naloxone. The results (Figure 6) showed that naloxone administration did not affect the recovery of the analgesic response to morphine.

Rate of loss of naloxone sensitivity in mice

Of the techniques available for assessing the degree of physical dependence in opioid-treated small

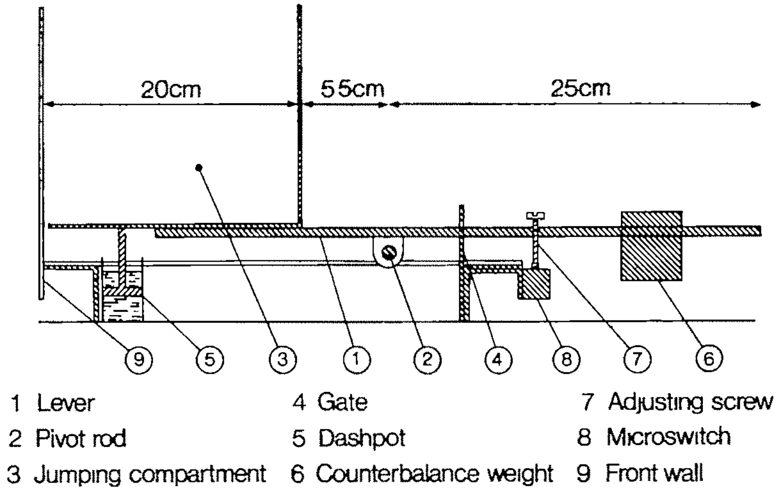

Figure 8 Counter for recording mouse jumping activity.

laboratory animals, the procedure which most readily yields quantitative data is the measurement of the incidence of the characteristic jumping (escape) response elicited by naloxone in dependent mice (Marshall & Weinstock, 1969; Cheney & Goldstein, 1971).

Experiments to compare the rate of loss of naloxone sensitivity with the rate of recovery of the analgesic response to morphine were carried out at various times after the cessation of pretreatment D (Table 1) On each day the tests for responsiveness to the antinociceptive effects of morphine HCl (12 mg/kg) given subcutaneously with cycloheximide (20 mg/kg) were followed 4-5 h later by an injection of naloxone HCl (10 mg/kg i.p.) and determination of the jumping activity over the ensuring 20 minutes.

Figure 7 shows, plotted on arithmetic scale, the loss of the naloxone response and the loss of tolerance to the antinociceptive effect of morphine as time elapses following the final dose of the morphine pretreatment, the former being expressed as the mean number of naloxone-elicited jumps per mouse and the latter in the increase in hot plate reaction time induced by morphine. Naloxone sensitivity declined much more rapidly than tolerance Thus on day 8, no jumps could be elicited by the naloxone treatment although the mice were still clearly tolerant to the analgesic effect of morphine.

Discussion

The rate of offset of morphine tolerance may be obtained by estimating at time intervals after cessation of pretreatment with morphine either, the recovery of the response to a fixed dose of morphine or the dose of drug required to elicit an arbitrarily defined standard response. For the present experiments the former strategy was adopted because it is more economical in time and in resources.

In general a pattern of change in one of these parameters will be reflected in the other provided that alteration in the position of the log dose-response curve is not accompanied by alteration of slope. This has been shown to be the case in the comparison of naive mice with mice two days after morphine withdrawal and in the contrast of naive rats with rats 7 days after morphine withdrawal. However, slight flattening of the log dose-response curve was observed after 2 days abstinence in rats, an effect which could be predicted on pharmacokinetic grounds from the persistence of some of the morphine from the tolerance-inducing treatment. Thus any discrepancy arising from this source would affect estimates of the recovery rate only in the period immediately following cessation of pretreatment, a period in which, for other reasons given in **Results**, no attempt was made to reach a precise estimate of recovery rate.

It should be remembered that owing to a restriction imposed by the experimental design (arising from avoidance of injurious nocioceptive stimuli), all the analgesic responses probably lie in the lower half of a complete log dose-response curve. Nevertheless, the smallest analgesic response considered by us to be significant was represented by points on the straight line portion of the curve.

In both rats and mice the return of the analgesic response to the standard morphine treatment occurred in two phases There was an initial rapid recovery which was complete in 3-4 days, followed by a slower phase. The half-time for this slower recovery phase ($T_{½}$ 13.2 days in rats, 17.4 days in mice) was independent of the intensity or means of the tolerance induction. These results differ from those reported by Goldstein & Sheehan (1969) on recovery from tolerance to the 'running-fit' caused by levorphanol in mice. They found that recovery occurred as a single phase, with a half-time of 16 h when tolerance-inducing doses of levorphanol were given at 8 h intervals, or with half-time of 48 h when the tolerance-inducing doses were given at 16 h intervals. Goldstein & Sheehan suggest that their results are consistent with the proposition that the opioid drug induces a change in the synthesis rate of an enzyme of functional protein maintained at steady state level by end-product control of the synthesis rate and an exponential rate of breakdown, although it is not clear why the recovery half-time should vary with the frequency of administration of the tolerance-inducing drug.

Our results on the recovery of the analgesic response are not consistent with this explanation since the half-time of slower recovery phase is considerably longer than the half-time for tolerance development (e.g. consider morphine pretreatment A which lasted for 3 days only). Recovery times greatly in excess of these have been described by Cochin & Kornetsky (1964) who reported that the analgesic response to morphine in rats still had not returned to normal 14 months after a single prior application of morphine (20 mg/kg). We cannot explain the discrepancy between their results and the progressive return over a period of 20-30 days to the sensitivity of naive animals, as described in this paper.

The contrast between the single phase rapid recovery of the 'running fit' response (Goldstein & Sheehan, 1969) and the more prolonged biphasic recovery of the analgesic response suggests that different processes are concerned in tolerance development with regard to these two opioid drug effects, or that a similar mechanism with a rapid recovery time is involved in tolerance to both effects but that an additional process with a slower recovery time is also involved in tolerance to the analgesic response.

The close agreement of the half-time values of the second recovery phase after morphine, diamorphine or methadone treatment, despite the differences in the manner in which these drugs are distributed within and eliminated from the body (Misra, 1972) and in their physical properties (Herz & Taschemacher, 1972) argues against the conclusion that the second phase reflects the time course of elimination of the opioid drug from the body and strongly reinforces the view that the nature of the persistent functional change underlying the morphine tolerant state is independent of the opioid drug used to induce that state. Furthermore, the failure of naloxone treatment to influence either the rapid or slow phases of recovery of the analgesic response to morphine also suggests that neither of these phases reflect the rate of dissociation of morphine-receptor complexes, since opioid antagonists are thought to compete with morphine for a common receptor site (Cox & Weinstock, 1964, Grumbach & Chernov, 1965, Pert & Snyder, 1973).

The rate at which naloxone-induced jumping activity in mice declined after the termination of the initial morphine treatment was considerably faster than the rate of loss of tolerance to the analgesic effects of morphine; Cheney & Goldstein (1971) also have reported a rapid dissipation of naloxone sensitivity in the mouse.

If there is a common mechanism associated with physical dependence and tolerance then it is characterized by a rapid relaxation ($T_{½}$ 3-6 h) whilst there is associated separately with tolerance to the analgesic action or morphine, an additional process with a half-time of about 17 days in mice and 13 days in rats It is probable that the latter reflects the reversal of an opioid-induced metabolic disturbance, and knowledge of its recovery rate may assist in identification of the processes involved.

Appendix

Counter for recording mouse jumping activity

The jumping counter (Figure 8) operates on a counter-balance principle. The lever, 1, is a flat perspex strip 3 cm wide which pivots about a rod, 2, and supports the floor and rear wall of the jumping compartment, 3, which are made of 1.5 mm white perspex sheet. The excursion of the lever assembly is limited by a gate, 4, and a dashpot, 5, is provided to dampen oscillations. The position of the counter balance weight, 6, is so adjusted that the weight of a mouse on the floor of the jumping compartment is just sufficient to depress that end of the lever assembly. When the mouse jumps the position of equilibrium of the lever shifts and the tip of the adjustment screw, 7, closes the microswitch, 8 (Honeywell B2-2RW84N27-D14/68) and activates a digital counter (Type E 350:ITT Electronic Services).

The dimensions of each jumping compartment are 20 x 15 x 30 cm high, the counter used in the experiments described above has five of these, mounted side-by-side. The three walls which enclose the moving parts of each compartment are also made of white perspex and are fixed to the chassis of the counter assembly. The front wall, 9, is hinged to facilitate cleaning the apparatus after use.

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ACTION OF β-ADRENOCEPTOR ANTAGONISTS ON THE RESPONSE TO ISOPRENALINE IN THE OESTROGEN DOMINATED RABBIT UTERUS

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1 Log dose-response curves to isoprenaline from spontaneously contracting muscle strips from rabbit uterus have been obtained. The effects of the α-adrenoceptor antagonists phentolamine and phenoxybenzamine, the β-adrenoceptor antagonists propranolol and practolol, and (+)-propranolol on the log dose-response curves were studied.

2 Phentolamine 5.3×10^{-7} M had a stimulating effect on the muscle strips, moving the log dose-response curve of isoprenaline to the right. Phenoxybenzamine 2.9×10^{-5} M had no effect on the curves.

3 Propranolol 3.4×10^{-6} M had no effect on the curves from circular muscle strips, with either phentolamine 5.3×10^{-7} M or with phenoxybenzamine 2.9×10^{-5} M as α-blocker. The curves from the longitudinal muscle strips were shifted somewhat to the right, the same shift, however, being obtained with (+)-propranolol 3.4×10^{-6} M.

4 Practolol 3.8×10^{-6}, $3 8 \times 10^{-5}$ and 3.8×10^{-4} M was without effect on the curves, either in the circular or in the longitudinal strips.

5 It is concluded that neither propranolol at 3.4×10^{-6} M nor practolol act as β-adrenoceptor antagonists in oestrogen dominated rabbit uterus. In all other tissues investigated, propranolol or practolol block the effect of isoprenaline. Whether the effect of isoprenaline in this tissue may be termed a β-effect, is then a question of definition. In addition there is no evidence for any extraneuronal uptake mechanisms for isoprenaline.

6 Variations in sensitivity to β-stimulation with the time of the year were observed, the sensitivity being greatest in the winter and lowest in the summer.

Introduction

The effects of stimulation of the α- and β-adrenoceptors in the rabbit myometrium are well known (for references, see Marshall, 1970). Stimulation of the β-adrenoceptors invariably causes inhibition of the spontaneous activity of the uterine smooth muscle. One of the procedures used for differentiating types of adrenoceptors is a quantitative determination of the capacity of an antagonist to reduce the sensitivity of the responding tissue to an agonist (Furchgott, 1967). Pharmacological testing of this type, involving different β-adrenoceptor antagonists, has again produced evidence for the existence of different types of β-adrenoceptors in different tissues (for review, see Furchgott, 1972). Previous *in vitro* experiments have shown (Nesheim, 1972) that the longitudinal and the circular muscle strips of the oestrogen-dominated rabbit uterus react differently to β-stimulation, the longitudinal muscle being much more sensitive to β-stimulation than the circular muscle. During these studies it was discovered that the inhibitory response to the β-adrenoceptor agonist isoprenaline could not be blocked to any great extent by the β-adrenoceptor antagonist propranolol.

The experiments presented here were undertaken in order to study in more detail the effect of β-adrenoceptor antagonists and, if possible, to decide to which group of β-adrenoceptors the ones of the rabbit myometrium belong.

Methods

Sixty-four rabbits, each weighing 2-4 kg, were used for the experiments. Ovariectomy was performed through incisions of about 2 cm in the flanks, after which a tablet of 25 mg diethylstilboestrol was implanted subcutaneously. One week later the animal was given a blow on the neck and thereafter bled to death by cutting the carotid arteries. The uterus was removed immediately and placed in modified Ringer solution as described previously (Nesheim, 1972). Nineteen rabbits were

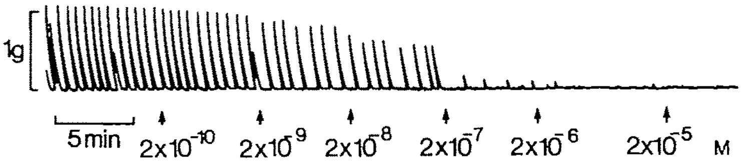

Figure 1 Tracing from an experiment on a longitudinal muscle strip from oestrogen dominated rabbit uterus Reserpine-treated rabbit. Phenoxybenzamine present in the bath. To the left: spontaneous activity immediately before the addition of isoprenaline At the arrows, isoprenaline was added to the bath.

given reserpine 1 mg/kg intraperitoneally or intravenously 24 h before they were killed.

Muscle strips, 10-15 mm long and 2 mm wide, were cut from the uterus in the longitudinal and the circular direction The strips were transferred to organ baths, as described by Nesheim (1972). Muscle tension was recorded isometrically. All strips were allowed to equilibrate in the bath for at least one hour. Adrenoceptor antagonists were added immediately after the strips were mounted.

The β-adrenoceptor agonist used was isoprenaline, at concentrations of 2×10^{-11} M-2×10^{-5} M. The dose-response curves were obtained by cumulative addition of isoprenaline, always starting with the lowest concentration and multiplying it by ten with each addition of the drug. The time interval between each addition of drug was six minutes. Each muscle strip was used only once.

The muscle strips had spontaneous activity. Isoprenaline inhibited this activity (Figure 1). The degree of inhibition of the spontaneous activity caused by the β-adrenoceptor agonist was calculated by the following procedure: the height of each contraction during one 5 min interval just before any drug had been added was measured and these heights were added together. Then the heights of each of the contractions in a 5 min interval after addition of each concentration of the drug were added together. The first minute after the addition of the drug was ignored in order to allow the drug to produce its effect. The difference between the sums obtained before any drug had been added and after addition of each concentration of the drug, expressed as percentage of the sum obtained before the addition of the drug, was used as a measure of the inhibition.

As stated previously (Nesheim, 1972), the responses to a certain concentration of isoprenaline varied a great deal, both within the same rabbit and between different rabbits. The variations in response between different muscle strips from one rabbit were smaller than the variations between the different rabbits. Therefore, the median of the responses from the individual muscle strips taken from each rabbit was used in the statistical calculations. The median of these medians is plotted in the figures.

The log dose-response curves were obtained by fitting the logistic function $y = Y \cdot x^n/(x^n + k^n)$ to the experimentally determined points by a non-linear least squares method (Parker & Waud, 1971). In this equation Y = maximum response, x = drug concentration giving response y, $k = ED_{50}$, n = a power determining the slope of the curve. During the fitting procedure, all three parameters Y, k and n could be varied, or any one or two of them could be kept constant.

The experiments were done in five series. In each series the effect of one of the antagonists was compared to controls in such a way that approximately the same number of muscle strips were used with antagonist and as controls from each animal. Each experimental series consisted of 10-12 rabbits, and each rabbit yielded 30-40 muscle strips.

To estimate the effect of the β-adrenoceptor antagonists each rabbit was considered separately. For each concentration of isoprenaline the median response of all muscle strips was taken. The median log dose-response curves obtained in this way, with and without β-adrenoceptor antagonist, were compared. In order to do this the two curves were fitted simultaneously with the same n and Y. Thus for each rabbit median curves with and without β-adrenoceptor antagonist were obtained, which gave the best fit to the experimentally determined points, given that the only effect of the drug was to change ED_{50}. The ratio of ED_{50} with and without antagonist was calculated for each rabbit. Three parameters characterizing the distributions of these ratios within each experimental series are shown in Table 2. These parameters are the median ($\tilde{x}$) and the upper and lower 90% confidence limits. When the value 1.0 is included between the upper and lower confidence limits, this means that the antagonist had no statistically significant effect.

As it could not safely be assumed that the distributions of the ED_{50} ratios were symmetrical, the method described by Noether (1971) was used to find the confidence limits. The statistical

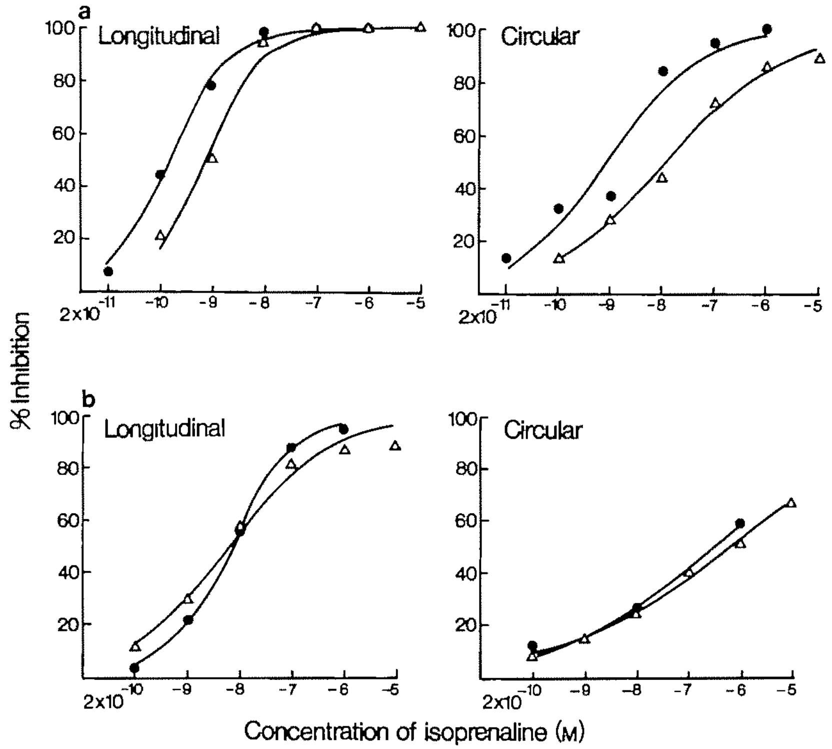

Figure 2 Log dose-response curves to isoprenaline in circular and longitudinal muscle strips from rabbit uterus. Abscissae concentration of isoprenaline (M). Ordinates: % inhibition of spontaneous activity. (a) With phentolamine 5.3×10^{-7} M added to the bath (△); without phentolamine (●). Median results from 12 rabbits. (b) With phenoxybenzamine 2.9×10^{-5} M added to the bath (△), without phenoxybenzamine (●). Reserpine-treated rabbits. Median results from 6 rabbits.

method used for determination of the significance of the difference between the experimental log dose-response curves was a one-sided Wilcoxon–van Elteren test (van Elteren, 1960), and for determination of the significance of the difference between any two points on the curves Wilcoxon two sample test was used.

The drugs used were: isoprenaline sulphate, phentolamine (Regitin), phenoxybenzamine, (±)-propranolol (Inderal), practolol (Eraldin), (+)-propranolol and reserpine (Serpasil).

Results

Isoprenaline had some α-activity in high concentrations. When no α-adrenoceptor antagonist was present in the bath, the stimulation caused by isoprenaline 2×10^{-5} M was similar to that of isoprenaline 2×10^{-4} M when the α-adrenoceptor antagonist was present. Lower concentrations were used to study the β-activity.

Effect of phentolamine

Figure 2a shows the effect of the presence of phentolamine 5.3×10^{-7} M in the organ bath on the log dose-response curves to isoprenaline. The muscle strips were less sensitive to isoprenaline in the presence of phentolamine ($\alpha < 0.001$). Phentolamine might have been expected to leave the curves unchanged or to have shifted them in the opposite direction if isoprenaline in the concentrations used had had any α-activity and phentolamine had blocked this activity. Phentolamine therefore must have stimulating properties on the rabbit myometrium.

When all three parameters Y, k and n were allowed to vary during the curve fitting, Y ended up close to 100%. During the fitting procedure of the curves plotted in Figure 2a, Y was kept constant at 100%. The same procedure was adopted for all subsequent curves where Y ended up close to 100% (Figures 2-4).

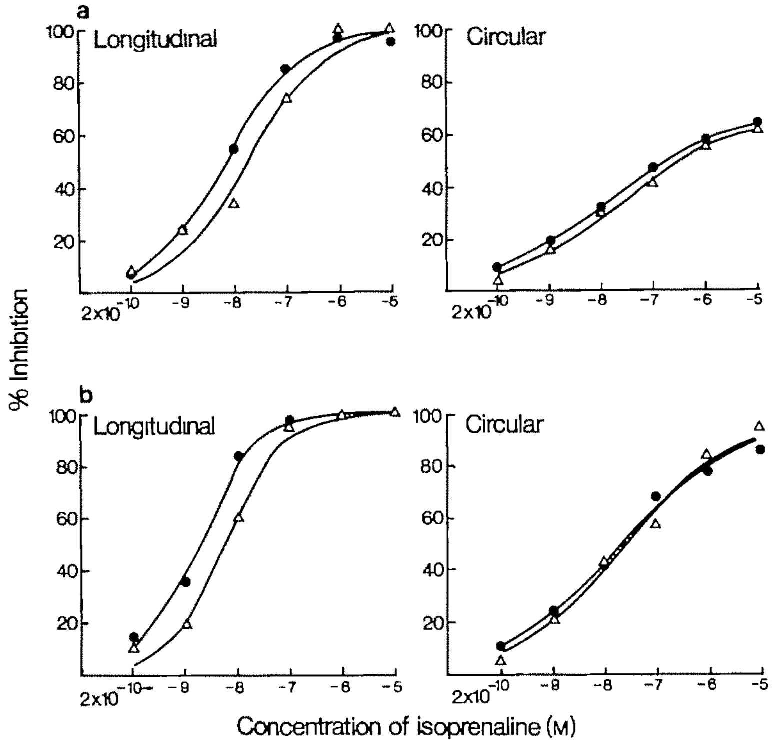

Figure 3 Abscissae and ordinates as in Figure 2 Phentolamine 5 3 x 10^{-7} ,M was always present in the organ bath (a) Controls (●), with (±)-propranolol 3 4 x 10^{-6} M (△). Median results from 12 rabbits. (b) Controls (●), with (+)-propranolol 3.4 x 10^{-6} M (△). Median results from 10 rabbits.

Effect of phenoxybenzamine

Since phenoxybenzamine can release endogenous catecholamines in some tissues (Furchgott, 1972) the experiments were done on reserpine-treated rabbits. Figure 2b shows the effect on the log dose-response curves to isoprenaline of phenoxybenzamine 2.9 x 10^{-5} M. The curves are fairly similar. There is no statistically significant difference between any two points on the curves when tested with a Wilcoxon two sample test.

Effect of (±)-propranolol with phentolamine as α-blocker

Three concentrations of (±)-propranolol were used: 2.0 x 10^{-6}, 2.7 x 10^{-6} and 3.4 x 10^{-6} M. The two lowest concentrations had little effect on the log dose-response curves, and complete series with these concentrations were therefore not carried out. Figure 3a shows log dose-response curves to isoprenaline with and without (±)-propranolol 3.4 x 10^{-6} M present in the bath. Phentolamine 5.3 x 10^{-7} M was used to block the α-adrenoceptors.

The curves from the circular strips were very similar and were not statistically significantly different. The curve from the longitudinal strips was shifted somewhat (statistically significant) to the right, the median ratio between the ED_{50} with and without (±)-propranolol being 2.4 (Table 2).

Effect of (+)-propranolol

Figure 3b shows log dose-response curves to isoprenaline with and without (+)-propranolol 3.4 x 10^{-6} M present in the bath. Phentolamine 5.3 x 10^{-7} M was used for blocking the α-adrenoceptors.

As for (±)-propranolol, the curves from the circular strips were almost identical. (+)-Propranolol shifted the curve from the longitudinal strips to the right (statistically significant), the median ratio between the ED_{50} with and without (+)-propranolol being 8.0 (Table 2).

Effect of practolol

Practolol in the concentrations 3.8×10^{-6}, 3.8×10^{-5} and 3.8×10^{-4} M was used. Phentolamine 5.3×10^{-7} M was used for blocking the α-adrenoceptors Tables 1 and 2 show that practolol did not move the curves significantly to the right, and larger concentrations of practolol did not give any increase in blocking effect.

Effect of (±)-propranolol with phenoxybenzamine as α-blocker

The rabbits were pretreated with reserpine. Figure 4 shows log dose-response curves to isoprenaline with and without (±)-propranolol 3.4×10^{-6} M. Phenoxybenzamine 2.9×10^{-5} M was always present in the organ bath The curves from the circular strips were almost identical, while in the longitudinal strips propranolol shifted the log dose-response curve to the right (statistically significant), the median ratio between the ED_{50} with and without (±)-propranolol being 7.0.

Variations in sensitivity with time of the year

In Figure 5 the log dose-response curves to isoprenaline from all the control curves from the experimental series described above are shown, with reference to the time of the year. For the sake of clarity only the curves from the circular strips are shown; the longitudinal strips showed exactly the same behaviour. In addition to the results from experiments already described one curve obtained from results with reserpine-treated rabbits is shown in Figure 5.

Table 1 Values of n, ED_{50} and Y obtained by fitting the experimental log dose-response curves to the logistic function $y - Y \cdot x^n/(n^n + k^n)$ (see text)

Curves from Figure	*Muscle and treatment*	*n*	*ED_{50}*	*Y*
2a	Longitudinal, without α-blocker	0.8	3.2×10^{-10}	
	Longitudinal, with phentolamine	0.8	1.5×10^{-9}	
	Circular, without α-blocker	0.5	1.9×10^{-9}	
	Circular, with phentolamine	0.4	2.5×10^{-8}	
2b	Longitudinal, without α-blocker	0.7	1.4×10^{-8}	
	Longitudinal, with phenoxybenzamine	0.5	1.2×10^{-8}	
	Circular, without α-blocker	0.3	6.5×10^{-7}	
	Circular, with phenoxybenzamine	0.3	1.3×10^{-6}	
3a	Longitudinal, control	0.6	1.4×10^{-8}	
	Longitudinal, with (±)-propranolol	0.6	3.5×10^{-8}	
	Circular, control	0.4	2.8×10^{-8}	68%
	Circular, with (±)-propranolol	0.4	4.2×10^{-8}	66%
3b	Longitudinal, control	0.8	3.2×10^{-9}	
	Longitudinal, with (+)-propranolol	0.8	1.1×10^{-8}	
	Circular, control	0.4	4.6×10^{-8}	
	Circular, with (+)-propranolol	0.4	5.6×10^{-8}	
	Longitudinal, control	0.8	1.5×10^{-9}	
	Longitudinal, with practolol 3.8×10^{-6} M	0.8	2.2×10^{-9}	
	Circular, control	0.4	2.5×10^{-8}	
	Circular, with practolol 3.8×10^{-6} M	0.4	5.4×10^{-8}	
	Longitudinal, control	0.8	2.6×10^{-9}	
	Longitudinal, with practolol 3.8×10^{-5} M	0.8	3.8×10^{-9}	
	Longitudinal, with practolol 3.8×10^{-4} M	0.7	1.2×10^{-8}	
	Circular control	0.4	6.6×10^{-8}	
	Circular, with practolol 3.8×10^{-5} M	0.4	9.7×10^{-8}	
	Circular, with practolol 3.8×10^{-4} M	0.3	1.8×10^{-7}	
4	Longitudinal, control	0.4	2.2×10^{-8}	
	Longitudinal, with (±)-propranolol	0.4	8.8×10^{-8}	
	Circular, control	0.3	8.7×10^{-7}	
	Circular, with (±)-propranolol	0.3	1.0×10^{-6}	

Where Y is not given in the table, it was kept constant at 100%.

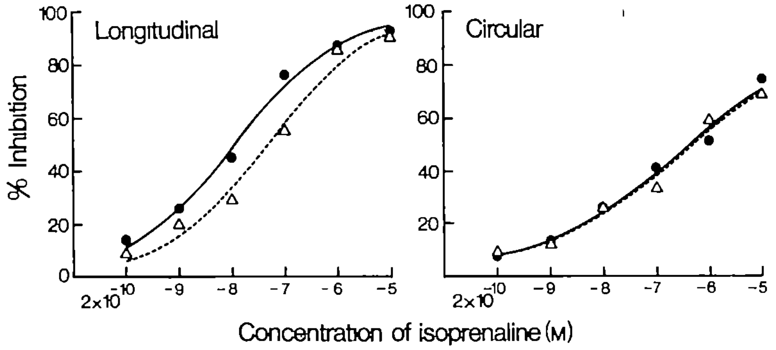

Figure 4 Abscissae and ordinates as in Figure 2. Phenoxybenzamine 2.9×10^{-5} M was always present in the organ bath. Controls (●), with (±)-propranolol 3.4×10^{-6} M (△). Reserpine-treated rabbits. Median results from 10 rabbits.

The muscle strips were most sensitive to inhibition of the spontaneous activity with isoprenaline during winter time, less sensitive in the spring and least sensitive during the summer. The differences between the curves are statistically significant ($\alpha = 0.05$ for the difference between the curves December-February 1972-73/ February-May 1973, $\alpha < 0.0001$ for the difference between the curves February-May 1973/March-June 1971).

Table 2 Median ratio of equiactive concentrations (X) of isoprenaline in presence and absence of a β-antagonist.

Muscle	*β-antagonist (M)*	*α-antagonist (M)*	*X*	*90% confidence limits*	*Number of animals*
Circular	(±)-propranolol $3.4\cdot10^{-6}$	phentolamine $5.3\cdot10^{-7}$	1.0	0.1-1.3	10
Circular	(±)-propranolol $3.4\cdot10^{-6}$	phenoxybenzamine $2.9\cdot10^{-5}$	1.1	0.8-3.0	10
Circular	(+)-propranolol $3.4\cdot10^{-6}$	phentolamine $5.3\cdot10^{-7}$	0.7	0.3-3.3	10
Longitudinal	(±)-propranolol $3.4\cdot10^{-6}$	phentolamine $5.3\cdot10^{-7}$	2.4	1.4-4.8	11
Longitudinal	(±)-propranolol $3.4\cdot10^{-6}$	phenoxybenzamine $2.9\cdot10^{-5}$	7.0	3.3-10.1	10
Longitudinal	(+)-propranolol $3.4\cdot10^{-6}$	phentolamine $5.3\cdot10^{-7}$	8.0	2.7-11.2	10
Circular	practolol $3.8\cdot10^{-6}$	phentolamine $5.3\cdot10^{-7}$	1.2	0.6-7.0	12
Circular	practolol $3.8\cdot10^{-5}$	phentolamine $5.3\cdot10^{-7}$	1.7	0.1-2.8	10
Circular	practolol $3.8\cdot10^{-4}$	phentolamine $5.3\cdot10^{-7}$	2.1	0.5-8.1	10
Longitudinal	practolol $3.8\cdot10^{-6}$	phentolamine $5.3\cdot10^{-7}$	3.4	2.5-4.0	12
Longitudinal	practolol $3.8\cdot10^{-5}$	phentolamine $5.3\cdot10^{-7}$	2.1	0.4-5.6	9
Longitudinal	practolol $3.8\cdot10^{-4}$	phentolamine $5.3\cdot10^{-7}$	0.8	0.3-1.2	9

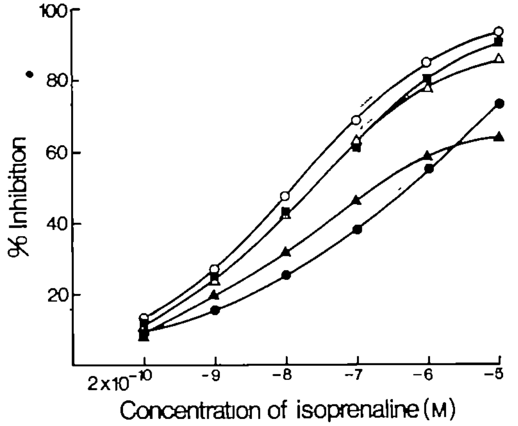

Figure 5 Variations in sensitivity with time of the year. Log dose-response curve to isoprenaline in circular muscle strips from rabbit uterus. December-February 1972-73 (○), February-May 1973 (■), January-March 1972 (rabbits were reserpine-treated) (△), March-June 1971 (▲), May-August, 1973 (reserpine-treated) (●) Phentolamine 5.3×10^{-7} M was present in the bath, except for (•), where phenoxybenzamine 2.9×10^{-5} M was used.

Discussion

This investigation has confirmed the difference between the circular and longitudinal muscle strips from the rabbit uterus in the reaction to stimulation of the β-adrenoceptors; even though the sensitivity of the strips varied with the time of the year, the longitudinal strips were always more sensitive than the circular ones (Nesheim, 1972).

In addition, it has been shown that phentolamine, but not phenoxybenzamine, reduces the sensitivity of muscle strips from the rabbit uterus to inhibition with isoprenaline. Phentolamine has been shown to have stimulating properties on muscle strips from rat uterus (Levy & Tozzi, 1963). It has never been shown to have any β-blocking activities. It is therefore reasonable to assume that the reduction in sensitivity to β-adrenoceptor stimulation by phentolamine seen in the experiments reported here was due to an unspecific stimulation of the muscle strips, although the spontaneous activity seemed to be of the same magnitude regardless of whether phentolamine was added to the bath or not.

Depending on the K_B (or pA_2) obtained with the different β-adrenoceptor antagonists in different tissues and on the relative potencies of agonists, there is evidence for multiple subgroups of β-adrenoceptors (for review and references, see Furchgott, 1972). The curves from the circular uterine muscle strips are not shifted at all with propranolol 3.4×10^{-6} M, and the shift that is obtained in the longitudinal strips is mimicked by the same concentration of (+)-propranolol. Both (±)- and (+)-propranolol are membrane stabilizers (Langslet, 1970). (+)-Propranolol has little, if any, β-blocking effects (Howe & Shanks, 1966). It therefore seems reasonable to assume that the shift in the log dose-response curve from the longitudinal strips was due to this unspecific membrane stabilizing effect of propranolol. It must be concluded that propranolol under these experimental conditions has no measurable β-blocking effect on the rabbit uterus. This fact places the β-adrenoceptors in the rabbit uterus in a group entirely different from those reported from other smooth muscle.

Practolol has less membrane stabilizing activity than propranolol (Hellenbrecht, Lemmer, Wiethold & Grobecker, 1973). It was therefore assumed that although practolol is a relatively selective cardiac β-adrenoceptor antagonist, its concentration might be high enough to confirm whether any β-blocking activity might be achieved at all in the rabbit uterus under these experimental conditions The log dose-response curves were not moved significantly to the right even with these high concentrations of practolol, and larger concentrations of practolol did not have any greater effect than lower concentrations. Thus, no β-blocking ability of practolol could be demonstrated in this preparation.

There is some evidence for an active extraneuronal uptake mechanism for isoprenaline in guinea-pig trachea (Foster, 1967, 1969), this uptake mechanism being inhibited by phenoxybenzamine. Furchgott (1972) has proposed that β-adrenoceptor agonists and antagonists might compete for such uptake mechanisms; in this way an erroneously high K_B would be produced. When phenoxybenzamine was used as a combined α-blocker and blocker of eventual uptake mechanisms in these experiments, the log dose-response curve to isoprenaline did not differ from the control curve without phenoxybenzamine, and the β-blocking effect of propranolol was no greater than when phentolamine was used for blocking the α-receptors. Thus no evidence has been produced for any uptake mechanisms for isoprenaline in the rabbit uterus.

The question may be raised whether the relaxation caused by isoprenaline in the spontaneously contracting rabbit uterus may be called a β-effect at all. The answer depends on how a β-effect is defined: if the definition includes the statement that the effect can be blocked by known β-adrenoceptor antagonists, the relaxation caused by isoprenaline in this preparation cannot be termed a β-effect In all instances where an effect has been thought to be mediated through β-adrenoceptors, the effect has been paralleled by a rise in cyclic adenosine 3′,5′-monophosphate. This is also the case in this preparation (Nesheim, Osnes & Øye, 1975) In this respect, the relaxation of the rabbit myometrium caused by isoprenaline is similar to β-effects in all other tissues.

Propranolol is a potent, nonselective β-adrenoceptor antagonist (Bristow, Sherrod & Green, 1970; Wasserman & Levy, 1972). It has been shown to antagonize the effects of isoprenaline in isolated uterus from the rat (Diamond & Brody, 1966; Wasserman & Levy, 1972), the mouse (Magaribuchi & Osa, 1971), and in man both in the pregnant and non-pregnant state (Stander & Barden, 1966, Sullivan & Marshall, 1970; Andersson, Ingemarsson & Persson, 1973, Lossius & Nesheim, unpublished results). The lack of β-blocking activity in the rabbit uterus described here, was therefore most surprising. Willems & de Schaepdryver (1966) reported an inhibition of the response of the oestradiol dominated rabbit uterus to isoprenaline by propranolol *in vivo*. They recorded the response from the longitudinal muscles only. In their experiments propranolol completely blocked the blood pressure response to isoprenaline, while the response from the uterus was diminished (not quantitated in the paper). It might well be that this diminished response was the same as that seen in the longitudinal muscle strips in these experiments, caused by the membrane stabilizing effect of propranolol.

Practolol has no β-blocking abilities in the rat (Wasserman & Levy, 1972) or the cat (Levy, 1973) uterus It was therefore not unexpected that practolol was also without antagonistic action in the rabbit uterus.

Variations with the time of the year of the sensitivity of the rabbit uterus to β-stimulation might be related to anovulation which has been observed from October to March, although the tendency is not absolute (Asdell, 1964). This is in very good agreement with the experience in this laboratory. However, this difference in sensitivity has no relevance to the other conclusions drawn from these experiments, since the log dose-response curves that are compared have always been obtained simultaneously from the same rabbits. It stresses the importance of making experiments at the same time of the year and, if possible, on the same animals when effects of adrenergic stimulation on smooth muscle from the reproductive tract are compared.

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ROLE OF CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE IN THE ISOPRENALINE-INDUCED RELAXATION OF THE OESTROGEN DOMINATED RABBIT UTERUS

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1 The role of cyclic adenosine 3′,5′-monophosphate (cyclic AMP) in the relaxation produced by isoprenaline in muscle strips from the oestrogen dominated rabbit uterus has been investigated.

2 Isoprenaline 2×10^{-8} M produced an inhibition of the mechanical activity but no increase in cyclic AMP. Isoprenaline 2×10^{-6} M produced both inhibition of mechanical activity and increase in cyclic AMP.

3 The increase in cyclic AMP, but not the inhibition of mechanical activity, was blocked by propranolol $3\,4 \times 10^{-6}$ M.

4 Dibutyryl-cyclic AMP produced a relaxation which mimicked that produced by isoprenaline, in that the longitudinal strips were more sensitive than the circular ones.

5 It is concluded that cyclic AMP may be a mediator of the β-adrenergic effect in the oestrogen dominated rabbit myometrium. However, it seems not to be an obligatory link between stimulation of β-adrenoceptors and relaxation. Other mechanisms may also exist.

Introduction

Strips from rabbit myometrium show spontaneous contractions *in vitro*. Isoprenaline inhibits this spontaneous activity (Nesheim, 1972). This inhibition is not blocked by either propranolol or practolol in concentrations which usually block β-adrenoceptors (Nesheim, 1975).

In all organ systems examined, stimulation of β-adrenoceptors is followed by an increase in the level of cyclic adenosine 3′,5′-monophosphate (cyclic AMP) in the tissue. A substantial amount of evidence now supports the hypothesis that β-adrenergic effects are mediated by cyclic AMP (Robison, Butcher & Sutherland, 1971).

It was therefore thought of interest to study the level of cyclic AMP in the rabbit myometrium where the mechanical effect produced by isoprenaline is not blocked by conventional β-adrenoceptor antagonists.

Methods

Seven rabbits were ovariectomized, and a tablet of 25 mg diethylstilboestrol was implanted subcutaneously. One week later the animals were killed and the uteri removed and stored in modified Ringer solution as described previously (Nesheim, 1972). Muscle strips 10-15 mm long and 2 mm wide, were cut in the circular and longitudinal direction, and transferred to organ baths containing the same solution at 39° C. The strips were allowed to equilibrate in the bath for one hour. Adrenoceptor antagonists were added immediately after the strips were mounted. Phentolamine $5\,3 \times 10^{-7}$ M was used for blocking α-adrenoceptors in all experiments.

Muscle tension was recorded isometrically, and the degree of inhibition calculated as described by Nesheim (1972).

For measurement of cyclic AMP the muscle strips were rapidly removed from the organ baths and frozen in liquid nitrogen.

Determination of cyclic adenosine 3′,5′-monophosphate

The frozen strips of uterus (average weight about 100 mg) were homogenized in 5% ice-cold TCA (19 µg per mg tissue weight) with a glass homogenizer. Hydrochloric acid was added to 1000 µl of the supernatant to give 0 1 M HCl. The sample was extracted 7 times with 2 ml ether and evaporated to dryness under a stream of air at room temperature. The dried extracts were dissolved in 300 µl 50 mM sodium acetate, pH 4.0, and cyclic AMP in 50 µl aliquots was determined by the method of Gilman (1970). The incubation volume was 200 µl. The millipore filters were dissolved directly in 10 ml of the scintillation fluid: BBOT-toluene-ethyleneglycolmonomethylether-naphthalene (4 g · 600 ml : 400 ml · 80 g). The cyclic AMP content was calculated as pmol/mg protein.

Determination of homogenate protein

Samples (50 μl) of the tissue TCA homogenate were dissolved in 550 μl 0.18 N NaOH, and the protein content was determined by the method of Lowry using bovine albumine as standard (Lowry, Rosebrough, Farr & Randall, 1951). The protein contents of the uterine strips were about 0.09-0.13 mg protein/mg tissue.

Drugs and chemicals

Isoprenaline sulphate, phentolamine (Regitin), propranolol (Inderal) (obtained through Norsk Medisinaldepot); [^{3}H]-cyclic 3',5'-AMP (24.1 or 22.1 Ci/mmol, New England Nuclear); cyclic 3',5'-AMP and bovine albumin (fraction V) (Sigma Chemical Co.); Folin-Ciocalten's phenol reagent (E. Merck, Darmstadt); 2,5-bis-(5'-*t*-butylbenzoxaxolyl (2'))-thiophene (BBOT), naphthalene, ethyleneglycolmonomethylether and toluene (Koch-Light Laboratories Ltd), N^6,O$^{2'}$-dibutryryladenosine 3',5'-cyclic monophosphate (D 0627, Sigma Chemical Company).

Results

Basal concentration of cyclic adenosine 3',5'-monophosphate

The basal concentration of cyclic AMP after equilibration for 1 h in the organ baths varied between the different rabbits, as shown in Table 1. Propranolol 3.4 x 10^{-6} M had no influence on the basal concentration of cyclic AMP

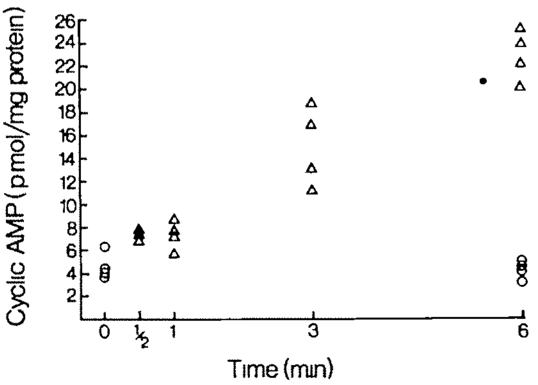


Figure 1 Concentration of cyclic 3',5'-adenosine monophosphate (cyclic AMP) in 24 myometrium strips from one rabbit (no 2, Table 1). At time 0, isoprenaline 2 x 10^{-6} M was added Controls (o) taken before addition of isoprenaline and 6 min after addition of an equal amount of water.

Effect of isoprenaline 2 x 10^{-8} M

Twenty-three circular muscle strips from one rabbit (rabbit no. 1 in Table 1), were examined. Twelve of the strips had been equilibrated with propranolol 3.4 x 10^{-6} M. Cyclic AMP was measured in controls, and 0.5, 1 and 6 min after addition of isoprenaline 2 x 10^{-8} M. In no instance was the concentration of cyclic AMP above the control level.

In the four strips examined after 6 min, the degree of inhibition of mechanical activity could be calculated. In one of the strips there was no inhibition, in the others the inhibition was 21, 27 and 73%, respectively.

Effect of isoprenaline 2 x 10^{-6} M

Figure 1 shows the concentration of cyclic AMP 0.5, 1, 3 and 6 min after the addition of

Table 1 Concentration of cyclic 3',5'-adenosine monophosphate (cyclic AMP) (pmol/mg protein) in rabbit uterine strips incubated for 1 h in aerated Ringers solution at 39°C with phentolamine 5.3 x 10^{-7} M and with or without propranolol 3.4 x 10^{-6} M.

Rabbit No.	*Propranolol*	*Number of muscle strips*	*Cyclic AMP (median)*	*Range*
1	–	3	2.2	1.2-2.3
	+	4	1.9	1.8-2.0
2	–	8	4.2	3.1-6.3
3	–	4	7.7	6.9-8.4
	+	4	7.2	6.6-7.8
4	+	8	4.6	4.1-5.4

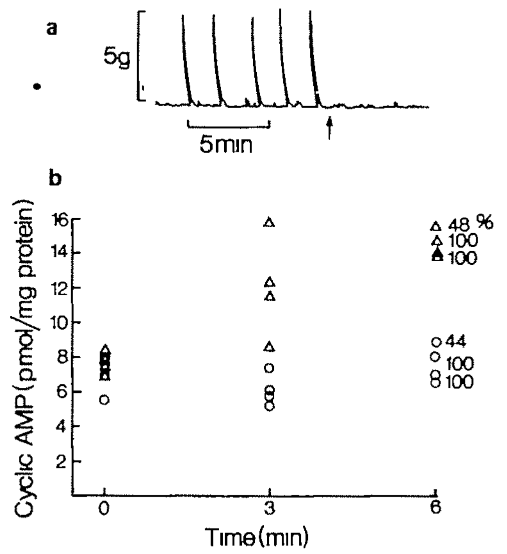


Figure 2 (a) Tracing from an experiment where propranolol 3.4 x 10^{-6} M was present in the bath. At the arrow, isoprenaline 2 x 10^{-6} M was added The concentration of cyclic adenosine 3',5'-monophosphate (cyclic AMP) in this particular strip is plotted as no. 2 from the bottom to the right in Figure 2b.
(b) Concentration of cyclic AMP in 24 myometrium strips in one rabbit (no. 3, Table 1). At time 0, isoprenaline 2 x 10^{-6} M was added. Controls were taken before addition of isoprenaline. Propranolol 3.4 x 10^{-6} M present in the bath (o), no β-blocker (△). Numbers to the right on the figure indicate % inhibition of mechanical activity in that particular muscle strip.

isoprenaline 2 x 10^{-6} M (rabbit no. 2 in Table 1). Controls taken before addition of isoprenaline and 6 min after the addition of an equal volume of water are included. All strips were cut circularly from the uterus. After 0.5 min the median concentration of cyclic AMP was 172% of the median control value ($\alpha = 0.002$, Wilcoxon's two sample test, one-sided), after 1 min 177% ($\alpha = 0.004$), after 3 min 359% ($\alpha = 0.002$) and after 6 min 552% ($\alpha = 0.002$).

The inhibition of the mechanical activity measured in the four strips where isoprenaline was allowed to act for 6 min, ranged from 54% to 79%. No correlation was found between the degree of inhibition of mechanical activity and the concentration of cyclic AMP.

Figure 2b shows the concentration of cyclic AMP in controls, and 3 and 6 min after the addition of isoprenaline 2 x 10^{-6} M with and without propranol 3.4 x 10^{-6} M present in the bath (rabbit no. 3 in Table 1). All were circular strips from the uterus. After 3 min, the median concentration of cyclic AMP without propranolol present, was 156% of the median control value ($\alpha = 0.01$, Wilcoxon's two sample test, one-sided), with propranolol 83% (not significantly different from control, $\alpha = 0.4$, two-sided test). After 6 min the concentration of cyclic AMP without propranolol was 188% ($\alpha = 0.01$, one-sided test), and with propranolol 104% (not significant, $\alpha = 0.6$, two-sided test).

The inhibition of the mechanical activity could be measured in 6 of the 8 strips where isoprenaline was allowed to act for 6 min (one strip with and one without propranolol did not have spontaneous activity). The inhibition of the mechanical activity for each particular muscle strip is indicated to the right in Figure 2b. Inhibition was the same in strips with and without propranolol, and there was no correlation between cyclic AMP concentration and inhibition of contractions. Figure 2a shows the tracing from an experiment with propranolol present in the bath, where the concentration of cyclic AMP was 6.9 pmol/mg protein (second point from bottom at 6 min in Figure 2b), and inhibition of mechanical activity was 100%.

To determine whether there might be a small rise in cyclic AMP 6 min after addition of isoprenaline when propranolol was present in the bath (104%, but not significantly different from control in Figure 2), additional experiments were done with another rabbit (rabbit no. 4 in Table 1). Strips were taken for cyclic AMP determination from controls, and 3 and 6 min after the addition of isoprenaline, with propranolol present in the bath Each group consisted of 8 muscle strips. After 3 min there was no rise in the cyclic AMP level. After 6 min there was a small rise, 109% of controls, which was statistically significant ($\alpha = 0.04$, two-sided test).

Effect of dibutyryl-cyclic AMP

The action of dibutyryl-cyclic AMP 5 x 10^{-5}, 10^{-4} 5 x 10^{-4} and 10^{-3} M was tested on 38 circular and 31 longitudinal muscle strips from 3 rabbits. Sodium-butyrate in the same concentrations was used as control in the same number of muscle strips. The effect of dibutyryl-cyclic AMP developed in 2-5 minutes. The mechanical activity was measured 10-15 min after addition of the drug, and compared to the mechanical activity during the last 5 min before addition of the drug.

Sodium butyrate inhibited the mechanical activity to a certain extent (median approximately 20%). The effect of each concentration of dibutyryl-cyclic AMP was calculated by subtracting the median inhibition caused by the same concentration of sodium butyrate from the median inhibition caused by dibutyryl-cyclic AMP.

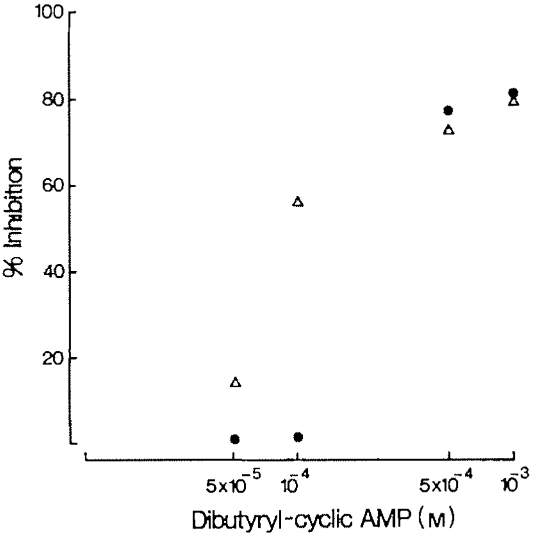


Figure 3 Inhibition of spontaneous mechanical activity in circular (●) and longitudinal (△) muscle strips from rabbit uterus by dibutyryl-cyclic AMP Abscissae. concentrations of dibutyryl-cyclic AMP (M) Ordinates: % inhibition of spontaneous activity. Each point represents the median of 6-14 individual muscle strips from one rabbit.

The values obtained in this way are plotted in Figure 3. For each concentration of dibutyryl-cyclic AMP, the values from the circular and longitudinal strips are from the same rabbit.

The significance of the difference from controls was tested with a one-sided Wilcoxon two sample test. For dibutyryl-cyclic AMP 5×10^{-5} M there was no inhibition of the circular strips, and the inhibition of the longitudinal strips was not significant at the 5% level ($\alpha = 0.08$). For 10^{-4} M there was no inhibition of the circular strips. The inhibition of the longitudinal strips was statistically significant ($\alpha = 0.0003$). For 5×10^{-4} M and 10^{-3} M all inhibitions were significant ($\alpha \leqq 0.006$).

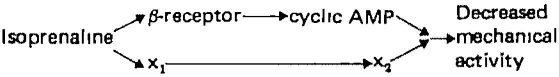
Discussion

Isoprenaline inhibited the mechanical activity and increased the cyclic AMP level in the oestrogen dominated rabbit uterus. This is in agreement with previous findings in other smooth muscle preparations (Robison *et al.*, 1971), including rat uterus muscle (Dobbs & Robison, 1968; Polacek & Daniel, 1971; Marshall & Kroeger, 1973). The levels of cyclic AMP 6 min after isoprenaline were quite different in the two rabbits tested (188% and 552%). The full effect of isoprenaline on the mechanical activity developed in the course of 1 min, and at this time cyclic AMP had increased to 177% of basal levels in the uterus where the ultimate level was 552%. The large increase in cyclic AMP content observed between 1 and 6 min was thus not associated with further inhibition of the contractile activity.

Under the conditions used, the inhibition of mechanical activity varied between different muscle strips and between different rabbits (Nesheim, 1972; 1975). The rise in cyclic AMP content showed much less variation between the different muscle strips of one uterus. No quantitative correlation was observed between the degree of inhibition of the mechanical activity and the rise of cyclic AMP. Because the longitudinal layer is more sensitive to isoprenaline than the circular layer (Nesheim, 1972; 1975), a strict quantitative correlation between the degree of mechanical inhibition and degree of rise in cyclic AMP content might be difficult to detect. In addition, only part of the cyclic AMP in each cell might be involved in regulation of contractile activity, which would also explain the different time-response relationships discussed above. Finally, the ability of the different muscle strips to respond to cyclic AMP might vary.

In the oestrogen dominated rabbit uterus we have found decreased mechanical activity without a measureable increase of cyclic AMP in two experimental situations: (1) with isoprenaline 2×10^{-8} M; (2) with isoprenaline 2×10^{-6} M in the presence of the β-adrenoceptor antagonist, propranolol. The ability of propranolol to block the isoprenaline-induced increase in cyclic AMP is in agreement with previous investigations in other smooth muscle preparations (Robison *et al.*, 1971). However, in the rabbit uterus the decrease of mechanical activity caused by isoprenaline was not affected by propranolol, as reported before (Nesheim, 1975). This is to our knowledge the first report on dissociation between decreased mechanical activity and cyclic AMP accumulation in uterine muscle after adrenoceptor stimulation. In the rat uterus, which has been most extensively studied, propranolol antagonizes both the mechanical and the metabolic effects of isoprenaline (Dobbs & Robison, 1968) or adrenaline (Polacek & Daniel, 1971).

Our findings may be interpreted in different ways: (1): The lack of correlation may reflect an apparent and not a functional dissociation. A local increase in cyclic AMP concentration sufficient to elicit the mechanical response could have escaped detection by the methods used. The small rise in cyclic AMP measured 6 min after the addition of isoprenaline with propranolol present in the bath, might support this interpretation. This rise might represent the 'overshoot' of cyclic AMP synthesis in a small intracellular compartment. The effect of dibutyryl-cyclic AMP strongly indicates an involvement of cyclic AMP in the decreased mechanical activity after β-adrenoceptor stimulation. (2): The results may represent a functional dissociation between cyclic AMP and decreased mechanical activity. Then two possibilities exist (a) cyclic AMP is not a mediator of adrenergic relaxation, (b) cyclic AMP is not an obligatory mediator; other mechanisms may contribute or even substitute for cyclic AMP under certain conditions. Confirmation of the former alternative would require the finding of a primary increase in cyclic AMP not accompanied by decreased mechanical activity. Such findings have never been reported. Polacek & Daniel (1971) observed a reversal of the inhibiting mechanical effects of isoprenaline in the rat uterus by addition of propranolol, without change in the elevation of cyclic AMP. This, however, might have represented a stimulation of α-receptors by isoprenaline in the presence of propranolol, as the experiments were performed without an α-blocker present. When intracellular cyclic AMP was elevated by addition of dibutyryl-cyclic AMP (Polacek & Daniel, 1971) decreased mechanical activity was observed. Our results with dibutyryl-cyclic AMP confirm these findings. Thus, alternative 2a above seems rather improbable, while alternative 2b accounts for the available information. This model includes a dual mechanism of the action of isoprenaline on the oestrogen-dominated rabbit uterine muscle:



This model has the following characteristics: cyclic AMP is involved as a mediator when it is increased; but it is not the only mediator and it is not an obligatory mediator. Cyclic AMP accumulation is blocked by propranolol, but the mechanical response is not affected. There is an alternative chain of processes leading to decreased mechanical activity. This mechanism is propranol-resistant. The common pathway beyond cyclic AMP may be responsible for the similar pattern of susceptibility of the longitudinal and circular muscle layers to both dibutyryl-cyclic AMP and isoprenaline. The nature of the non-cyclic AMP chain is unknown.

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INHIBITION OF IMMEDIATE HYPERSENSITIVITY REACTIONS IN LABORATORY ANIMALS BY A PHENANTHROLINE SALT (ICI 74,917)

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1 The activity of a new anti-allergic compound, I.C.I. 74,917, has been studied in the rat, mouse and guinea-pig.

2 Following intravenous administration, I.C.I. 74,917 inhibits in a dose-dependent manner passive cutaneous anaphylaxis induced in rats and mice by heat-labile homocytotropic antibody. In rats, its potency is approximately 300 times that of disodium cromoglycate.

3 To achieve maximal inhibition, it is necessary to administer I.C.I. 74,917 at the same time as antigenic challenge; dosing before or after challenge has much less effect.

4 Liberation of histamine, provoked by the antigenic challenge of mast cells passively sensitized *in vitro* by IgE-like antibody, is reduced in the presence of I.C.I. 74,917.

5 Intravenous administration of the compound has no significant effect upon local blueing reactions provoked in the rat by intradermal injection of histamine, 5-hydroxytryptamine or Compound 48/80. It has only a slight effect at high doses upon passive cutaneous anaphylaxis induced in the rat by heat-stable homocytotropic or heterologous (guinea-pig) antibodies.

6 Although not a bronchodilator in the guinea-pig, I.C.I. 74,917 partially inhibits systemic anaphylaxis. A consistent reduction in the severity of antigen-induced bronchospasm was demonstrated in the Konzett-Rössler preparation at doses comparable to those inhibiting passive cutaneous anaphylaxis in the rat. However, there was only slight inhibition of passive cutaneous anaphylaxis in the guinea-pig.

7 I.C.I. 74,917 itself induces bronchospasm when administered to anaesthetized guinea-pigs or to a guinea-pig isolated lung preparation. This effect is reversed by salbutamol, but is not prevented by the prior administration of mepyramine, atropine or methysergide.

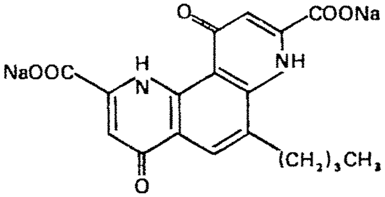
8 These results indicate that in the rat, mouse and guinea-pig, I.C.I. 74,917 is a potent inhibitor of certain types of immediate hypersensitivity reactions.

Introduction

Among the most significant advances in the last decade in the treatment of asthma were the discovery (Cox, 1967) of disodium cromoglycate (DSCG) and its introduction into clinical practice by Howell & Altounyan (1967).

Experimental models demonstrated that DSCG inhibited immediate (Type I) hypersensitivity reactions *in vivo* (Goose & Blair, 1969) and *in vitro* (Sheard & Blair, 1970). These studies suggested that the compound specifically inhibits reactions involving the heat-labile homocytotropic antibody (IgE-like), but more recent work (summarized by Cox, 1971) suggests DSCG is specific for a cell type, the mast cell, rather than for a particular class of immunoglobulin. Even so, the evaluation of DSCG-like compounds *in vivo* depends largely upon their ability to inhibit certain immediate hypersensitivity reactions in suitable laboratory animals. In rats, three models of immediate hypersensitivity reactions may be used. Of these passive cutaneous anaphylaxis (PCA; Ovary, 1964) induced by the IgE-like antibody is a particularly pertinent model as there is now little doubt that in man IgE plays a major role in the pathogenesis of certain types of asthma and hay fever (Ishizaka & Ishizaka, 1970; Johansson, Bennich & Berg, 1972). PCA may also be provoked by a heat-stable homocytotropic antibody, an IgG type (Stechschulte, Austen & Bloch, 1967), but the involvement of human IgG in the pathogenesis of asthma has not yet been unequivocally established (but see Parish, 1973). PCA can also be induced by heterologous antibody, probably reflecting the participation of a Type III (Arthus) reactivity. This type of response may be analogous to that which, in man, is responsible in the lung for allergic reactions of the immune complex type (Pepys, 1973).

Some chemical types other than the chromone-2-carboxylic acids, of which DSCG is an example, also inhibit rat PCA mediated by an IgE-like antibody, e.g. xanthone-2-carboxylic acids (Pfister, Farraresi, Harrison, Rooks, Roszkowski, Van Horn & Fried, 1972) and some 2-nitroindan-1,3-diones (Buckle, Morgan, Ross, Smith & Spicer, 1973). In this paper, we describe the anti-allergic properties in the rat, guinea-pig and mouse of a novel compound, 6-*n*-butyl-2,8-dicarboxy-4,10-dioxo-1,4,7,10-tetrahydro-1,7-phenanthroline (I.C.I. 74,917) as the disodium salt.



I.C.I. 74,917, disodium salt

A preliminary report on this compound has already been published (Evans, Gilman, Thomson & Waring, 1974).

Methods

The guinea-pigs, rats and mice used in this study were from closed colonies (random-bred) of S.P.F. albino strains maintained at Alderley Park for more than 10 years. The animals were housed under standard conditions and allowed free access to food and water.

Administration of drugs

DSCG and I.C.I. 74,917 were dissolved in 0.9% w/v NaCl solution (saline) or buffer as described in the text. Doses and concentrations are expressed in terms of the free acid of both compounds.

Production of antisera

Rat sera containing the heat-labile homocytotropic antibody (IgE-like) against egg albumin (EA; 2 x crystallized, Koch-Light Ltd) were prepared as described by Mota (1964) using *Bordetella pertussis* vaccine (4×10^{10} organisms/ml; Burroughs Wellcome & Co.) as adjuvant. When a 48 h latent period was used, these sera gave in the rat PCA titres between 1 : 8 and 1 : 32. Serum dilutions were chosen so that DSCG at a dose of 2.5 mg/kg intravenously produced a 50% inhibition of PCA; this dilution was usually between 1 : 2 and 1 : 4. Sera containing IgE-like antibody against the nematode parasite *Nippostrongylus brasiliensis* were produced in rats by repeated infection as described by Ogilvie (1967). With a 48 h latent period, a typical serum had a PCA titre of 1 : 640. It was employed at a dilution of 1 : 5 after being standardized against DSCG in the same way as anti-EA serum. The presence of IgE-like antibody was established by the persistence of PCA activity after a latent period in excess of seven days and by lability to heat and mercaptoethanol. Rat sera containing the heat-stable homocytotropic antibody (IgG) were prepared against EA as described by Davies & Evans (1973) using Freund's Complete Adjuvant (Difco). The sera were heated at 56° C for 2.5 h to destroy any IgE-like antibody present. With a 3 h latent period, the sera had a PCA titre of between 1 : 16 to 1 : 32 and were used at a dilution of 1 : 4. Guinea-pig sera were prepared as described by Davies & Johnston (1971) using EA and Freund's Complete Adjuvant.

Passive cutaneous anaphylaxis in the rat

Groups of five rats (150 to 180 g) of either sex were used. The technique was that of Mota (1964) with minor modifications. A latent period of 48 h was used with the IgE-like antibody and of 3 h for the IgG and heterologous (guinea-pig) antibodies. The severity of the PCA reactions was assessed by scoring in arbitrary units, 10 representing maximal severity (an intense blue reaction with a diameter in excess of 2 cm) and zero representing complete inhibition.

Drugs were administered intravenously as solutions in isotonic saline at the same time as antigenic challenge, unless otherwise stated in the text.

Local blueing reactions in the rat

Groups of five rats (150 to 180 g) of either sex received intradermal injections of histamine acid phosphate (BDH Ltd; 100 μg as the base in 0.1 ml of isotonic saline), 5-hydroxytryptamine creatinine sulphate (Sigma Chemical Co. Ltd; 10 μg as the base in 0.1 ml of isotonic saline) or Compound 48/80 (Burroughs Wellcome Ltd; 10 and 1 μg in 0.1 ml of isotonic saline). Drugs were administered intravenously, dissolved in saline immediately before the intradermal injections. In one series of experiments either DSCG or I.C.I. 74,917 in isotonic saline (0.05 ml) was administered intradermally immediately following and at the same site as Compound 48/80 (10 and 1 μg in 0.05 ml of isotonic saline). The severity of the reactions was assessed 30 min later as previously described.

Histamine release from rat peritoneal mast cells

Peritoneal cells were harvested from normal rats, washed, and sensitized by incubation with rat serum containing IgE-like antibody (Thomson & Evans, 1973). After thorough washing to remove excess antibody, the cells were challenged with EA in the presence or absence of drug as appropriate and the histamine liberated from the mast cells estimated by an automated assay (Evans, Lewis & Thomson, 1973) based on the fluorimetric procedure of Shore, Burkhalter & Cohn (1959).

The reduction in histamine release in the drug-treated groups is expressed as a percentage of the release in control groups receiving antigen alone. Some 50 to 60% of the net available histamine was released by antigen from these control cells.

Systemic anaphylaxis in the guinea-pig

Groups of female guinea-pigs (250 to 300 g) were sensitized by the intraperitoneal injection of alum-precipitated EA (36 mg). After three weeks, each animal received an intraperitoneal injection of mepyramine maleate (0.1 mg/kg) (May & Baker Ltd), to reduce the severity of anaphylaxis at subsequent challenge. One hour later, antigen (1 mg EA) and drugs were administered intravenously. The animals were then observed and the time of death recorded.

Allergic bronchospasm in the guinea-pig

Guinea-pigs were passively sensitized by intravenous injection of 0.5 mg of a 1 : 50 dilution of guinea-pig anti-EA antiserum. Twenty-four hours later they were anaesthetized with pentobarbitone sodium (70 mg/kg) deeply enough to depress spontaneous respiration. Cannulae were tied into the trachea and jugular vein, and the lungs inflated from a pump at a rate of 68 strokes/min at a constant stroke volume of between 6 and 8 ml. Resting pressure was recorded for 5 min with the apparatus described by Konzett & Rössler (1940) as modified by Davies (1973); following the suggestion of Collier & James (1967), the lungs were over-inflated for 10 s in every 30 seconds. Drug and antigen (1 mg) were then injected into the jugular vein cannula and recording continued for a further 15 minutes.

Passive cutaneous anaphylaxis in the guinea-pig

Groups of five animals including both sexes (300-350 g) were sensitized by an intradermal injection of heat-stable guinea-pig anti-EA antiserum diluted 1 : 1000. Three hours later, the animals were challenged intravenously with antigen (1 mg in 0.5 ml of isotonic saline). Evans blue dye (5% w/v : 0.5 ml) and drug were injected into the ear vein at the same time and the animals killed 30 min later. The severity of the response was assessed by measuring the maximum diameter of the blued areas.

Bronchospasm in the perfused isolated lungs of the guinea-pig

The method was that of Davies (1973). Normal guinea-pigs were killed by a blow on the head and the heart and lungs removed. After removal of the pericardium, a cannula was tied into the pulmonary artery and 10-20 ml of warmed Tyrode solution injected to remove most of the blood, the effluent being allowed to escape through an incision in the auricle. A second cannula was tied in the trachea and the preparation mounted in the apparatus described by Bhattacharya & Delaunois (1955). This apparatus consists essentially of a cylinder made of Perspex closed at the upper end by a lid through which pass tubes connected to the tracheal and arterial cannulae respectively. The lower end of the cylinder is attached to a small bellows pump delivering 7 ml of air per stroke at a rate of 25 strokes/minute. A small flap-valve allows the escape of air. A pressure transducer was attached to a side arm of the tube leading to the tracheal cannula. Compression of air within the cylinder deflates the lungs and withdrawal of air inflates the lungs, the resulting pressure-changes being monitored by the transducer which activates a pen-recorder, thus providing a record of the course of artificial respiration. An aspirator containing Tyrode solution with 2% dextran is attached, via a warming coil, to the arterial cannula, and the vascular system perfused at a rate of 2-3 ml/min, the effluent being allowed to escape through the flap-valve as it opens during the compression stroke of the pump.

I.C.I. 74,917 and other drugs were injected as appropriate into the perfusion fluid close to the arterial cannula and the degree of bronchospasm was estimated from the percentage change in the height of the recording.

Histamine release from guinea-pig lung

The method was a modification of that described by Colquhoun & Brocklehurst (1965). The chopped lung was divided into 300 mg (wet weight) replicates and 4 ml of guinea-pig anti-EA serum, diluted 1 : 60, added. The tissue was sensitized for 2 h at 37° C, washed twice with Tyrode solution and finally resuspended in 4 ml of the same solution but containing EA (100 μg/ml) and, if appropriate, I.C.I. 74,917. Fifteen min later, the supernatants were removed and 0.15 ml of 9 N perchloric acid added to them Four ml of 0.4 N perchloric acid was added to the lung fragments and each sample placed in a boiling water bath for approximately 15 minutes. Histamine content was determined as before.

Passive cutaneous anaphylaxis in the mouse

Groups of five mice (20 to 25 g) including both sexes were used (Mota, 1967). Each animal was given an intradermal injection (0.02 ml) of antiserum and challenged 48 h later by an intravenous injection (0.5 ml) containing antigen (2 mg/ml), Evans Blue (0.25% w/v) and, if appropriate, DSCG or I.C.I. 74,917. The severity of the blueing reaction was assessed 30 min later, as previously described

Results

Passive cutaneous anaphylaxis in the rat

Both I.C.I. 74,917 and DSCG inhibited PCA induced by the IgE-like antibody in a dose-dependent manner (Table 1). The ID_{50}, i.e. the dose required to reduce the PCA response of a group by 50%, was the same in both antigen systems, within the limits of experimental variation; I.C.I. 74,917 was approximately 300 times as potent as DSCG in both systems. To determine whether I.C.I. 74,917 would inhibit PCA if given before or after antigenic challenge, I.C.I. 74,917 (0.05 mg/kg i.v.) was administered to groups of five rats at various times before, and in one case after, antigenic challenge. The results demonstrated the necessity for simultaneous administration of drug and antigen to achieve maximal inhibition (Figure 1).

In contrast to the inhibitory activity in IgE-mediated PCA, neither compound induced a regular dose-dependent inhibition of PCA provoked by the heat-stable IgG antibody or by the heterologous (guinea-pig) antibodies. I.C.I. 74,917 at doses higher than 0.025 mg/kg i.v. and DSCG at doses higher than 5 mg/kg i.v. showed a partial inhibition (20 to 30%) of both responses which was statistically significant ($P \leqslant 0.05$), but increasing the dose did not result in enhanced inhibition

Dermal blueing reactions in the rat

Cyproheptadine, a potent antagonist of histamine and 5-hydroxytryptamine (5-HT), inhibited in

Table 1 Effect of I.C.I. 74,917 and disodium cromoglycate (DSCG) on rat passive cutaneous anaphylaxis (PCA) provoked by IgE-like antibody.

Compound	*Dose (mg/kg i.v.)*	*No. of animals*	*Mean score (± s.e. mean)*	*% inhib. (± s.e. mean)*	*ID_{50} *(mg/kg) (± 95% C.L.)*	*Relative potency (±95% confidence limits)*
(a) Egg albumin system:						
Control		75	9.6 ± 0.1			
DSCG	1.0	25	8.6 ± 0.2	11 ± 2		
	2.5	25	5.8 ± 0.3	39 ± 3	2.8 ± 0.2	1.0
	5.0	25	3.8 ± 0.3	61 ± 3		
I.C.I.	0.005	25	6.8 ± 0.3	29 ± 3		
74,917	0.01	25	4.6 ± 0.3	52 ± 3	0.009 ± 0.0005	323 ± 30
	0.025	25	3.2 ± 0.2	66 ± 2		
(b) Nippostrongylus brasiliensis *system:*						
Control		25	9.8 ± 0.1			
DSCG	1.0	20	8.1 ± 0.4	18 ± 4		
	2.5	20	5.7 ± 0.5	42 ± 5	3.4 ± 0.2	1.0
	5.0	20	2.9 ± 0.5	71 ± 5		
I.C.I.	0.005	20	7.3 ± 0.4	26 ± 4		
74,917	0.01	20	3.7 ± 0.4	62 ± 4	0.01 ± 0.0006	308 ± 24
	0.025	20	1.5 ± 0.3	85 ± 3		

*The ID_{50} is that dose required to inhibit the PCA response by 50% compared to an untreated control group.

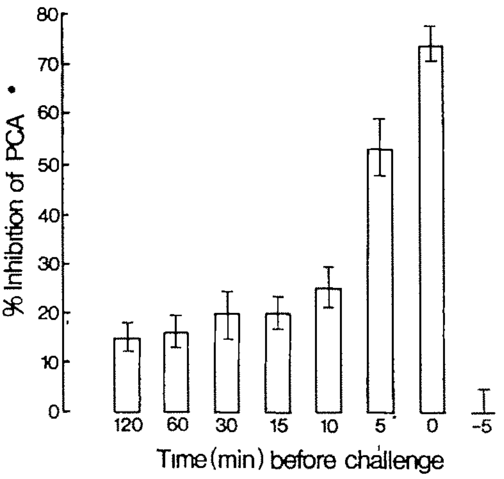


Figure 1 Influence of time of administration of I.C.I. 74,917 on inhibition of passive cutaneous anaphylaxis (PCA). Drug (0.05 mg/kg i.v.) was administered at various times before or, in one case, after antigenic challenge. Results are the mean from five rats, and are expressed in terms of % inhibition compared to an untreated control group. Vertical bars show s.e. mean.

intravenous doses of 0.25 to 1 mg/kg, in a regular, dose-dependent manner the blueing reactions induced by intradermal injection of histamine (100 μg), 5-HT (10 μg) and Compound 48/80 (0.1 and 10 μg). Neither DSCG nor I.C.I. 74,917 administered intravenously had any significant effect at 10 times the ID_{50} in rat PCA. In contrast, both drugs, administered intradermally, inhibited in a dose-dependent manner blueing reactions provoked by Compound 48/80 (Table 2). Although it was not possible to calculate a meaningful ID_{50} for each compound from these data, I.C.I. 74,917 was approximately 100 times more potent in this model than DSCG.

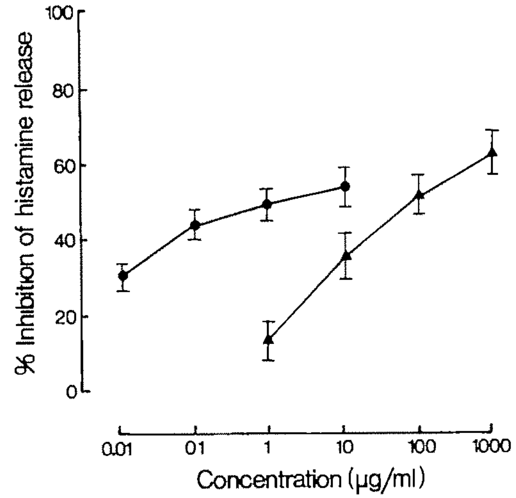


Figure 2 Inhibition of allergic histamine release from passively sensitized rat peritoneal mast cells by I.C.I. 74,917 (●) and disodium cromoglycate (▲). Results are the mean from seven and six experiments, respectively. Vertical bars show s.e. mean.

Histamine release from peritoneal mast cells

Both DSCG and I.C.I. 74,917, when added to sensitized peritoneal mast cells simultaneously with antigen, inhibited allergic histamine release (Figure 2). The dose-response curve for DSCG was steeper and more linear. The effect of I.C.I. 74,917 'levelled off' at approximately 50% inhibition, but for lesser degrees of inhibition this compound, as in the rat PCA test, is clearly much more potent than DSCG.

Allergic reactions and bronchospasm in the guinea-pig

Preliminary experiments established that I.C.I.

Table 2 Inhibition following intradermal administration of disodium cromoglycate (DSCG) and I.C.I. 74,917 of blueing reactions in the rat provoked by Compound 48/80.

Compound	*Total dose administered (μg)*	*No. of animals*	*% inhib. of blueing response (± s.e. mean)*	
			*10 μg**	*0.1 μg**
DSCG	50	15	5 ± 5.9	16 ± 7.3
	250	15	21 ± 3.6	33 ± 3.8
	500	15	32 ± 6.7	40 ± 6.2
I.C.I. 74,917	0.5	15	21 ± 4.1	25 ± 4.6
	5	15	38 ± 5.4	41 ± 5.5
	50	15	53 ± 3.7	59 ± 3.1

*Dose of Compound 48/80 injected per site.

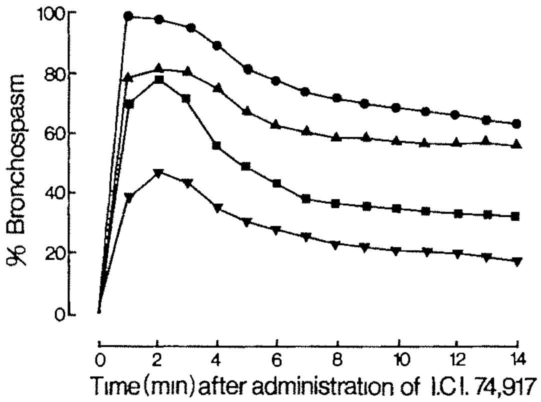


Figure 3 Inhibition of allergic bronchospasm in the anaesthetized guinea-pig Results are the mean from. untreated controls (●) 20 animals, I.C.I 74,917-treated, 10 animals/group, (▲) 2 5 μg/kg i.v., (■) 5 μg/kg i.v and (▼) 10 μg/kg i v.

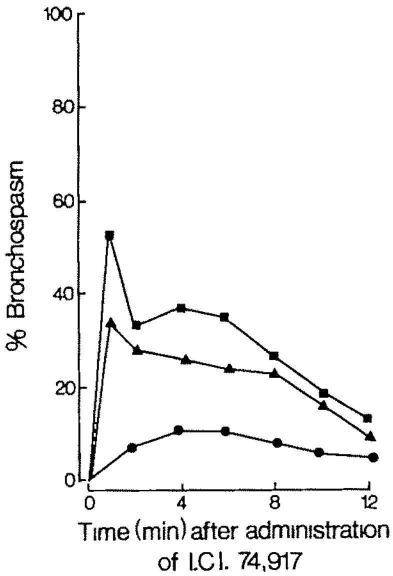


Figure 4 Induction of bronchospasm in anaesthetized guinea-pigs (Konzett-Rössler preparation) by I.C I. 74,917 Each curve represents the mean measurements from five animals after i.v. administration of 500 μg/kg (■), 50 μg/kg (▲), or 5 μg/kg (●) of compound.

74,917 at doses up to 10 mg/kg intravenously and DSCG at doses up to 25 mg/kg intravenously did not prevent or delay the onset or severity of dyspnoea or collapse provoked by inhalation of an aerosol of histamine or acetylcholine. In contrast, the β-adrenoceptor stimulants isoprenaline and salbutamol showed significant activity in both these models. I.C.I. 74,917 did, however, confer some protection against systemic anaphylaxis (Table 3), but only at doses higher than those inhibiting PCA in the rat; no dose-response relationship was evident. To study this apparent anti-allergic activity more quantitatively, allergic bronchospasm in the anaesthetized guinea-pig (the Konzett-Rössler preparation) was employed (Figure 3). Both the severity of the initial spasm and the duration of the subsequent protracted spasm were reduced in a dose-dependent manner by I.C.I. 74,917. The doses used were comparable to the doses active in rat PCA, but the effect could not be enhanced by employing higher doses (up to

Table 3 Inhibition of allergic reactions in the guinea-pig by I.C.I. 74,917.

(a) *Active systemic anaphylaxis*

Treatment	*Dose mg/kg i.v.*	*No. of animals*	*No. of survivors at 10 min*
Control		28	3
I.C.I. 74,917	0.01	10	7
	0.1	28	16
	1.0	28	14

(b) *PCA (heat-stable antibody)*

Treatment	*Dose (mg/kg i.v.)*	*No. of animals*	*Mean diameter (mm ± s.e. mean)*	*% inhib. (± s.e. mean)*
Control		10	16.2 ± 0.9	
I.C.I. 74,917	0.25	9	11.7 ± 1.2	28 ± 8
	0.5	10	8.5 ± 1.0	48 ± 7
	1.0	10	10.1 ± 0.7	38 ± 6

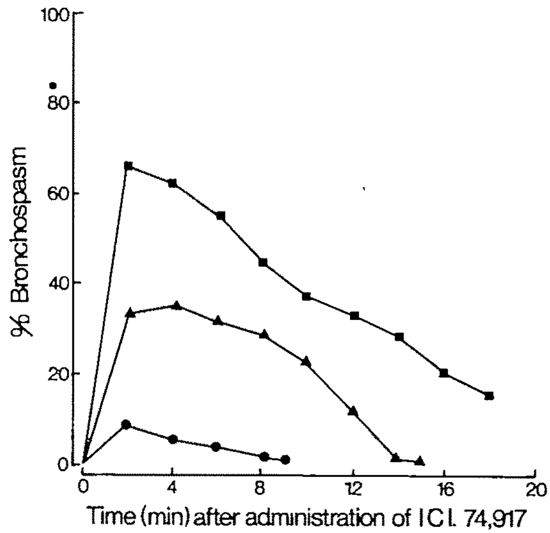


Figure 5 Induction by I.C.I 74,917 (●) 0.25 μg; (▲) 1 μg; and (■) 5 μg of bronchospasm in the isolated, perfused lung of the guinea-pig Each curve represents the mean measurements from five preparations.

500 μg/kg) of I.C.I. 74,917. Further experiments have shown that to achieve maximal inhibition at a given dose, it is necessary, as in rat PCA, to give drug and antigen simultaneously.

I.C.I. 74,917 itself induced mild reversible bronchospasm both in the anaesthetized guinea-pig (Figure 4) and in isolated perfused lung preparations. As little as 0.25 μg of I.C.I. 74,917/lung produced (Figure 5) a slight but measurable bronchospasm, and 5 to 10 μg produced a maximal response. Increases in dosage of I.C.I. 74,917 did not consistently result in a greater degree of bronchospasm.

When included in the perfusion fluid, mepyramine (1 μg/ml), atropine (1 μg/ml) and methysergide (25 μg/ml) antagonists of histamine, acetylcholine and 5-HT, respectively, did not affect the bronchospasm induced by I.C.I. 74,917. Furthermore, this response was not modified in any way by injection close to the arterial cannula of propranolol (50 μg) or the histamine H_2-receptor blocker, burimamide (1 mg). Bronchospasm initiated by I.C.I. 74,917 was rapidly reversed by injection of salbutamol into the cannula 2 min later (Figure 6).

Guinea-pig PCA was also inhibited by I.C.I. 74,917 (Table 3) but the doses (0.25 to 1 mg/kg i.v.) required were much higher than those inhibiting bronchospasm, and no dose-response relationship was found. In doses up to 50 mg/kg intravenously, DSCG had no activity on these immediate hypersensitivity reactions in the guinea-pig.

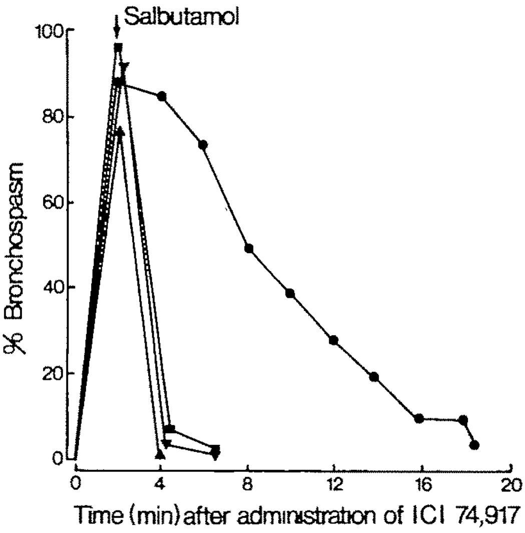


Figure 6 Reversal by salbutamol of bronchospasm induced by I C.I. 74,917 (10 μg) in the isolated perfused lung of the guinea-pig Each curve represents the mean measurements from five lung preparations I.C I. 74,917 was given alone (●), or followed 2 min later by 0.5 μg (▲); 0.25 μg (■); or 0.1 μg (▼) of salbutamol.

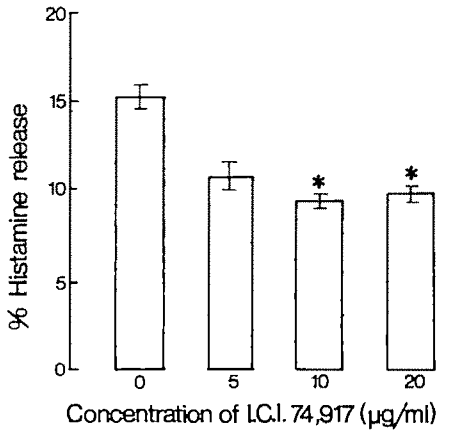


Figure 7 Reduction by I.C.I. 74,917 of histamine release from the chopped, sensitized, guinea-pig lung challenged *in vitro* with antigen. Results are pooled from two experiments performed in triplicate. Vertical bars show s.e. mean. Non-specific release was in all cases less than 1%. * Significant difference from controls ($P < 0.05$).

Preliminary experiments showed that I.C.I. 74,917 in concentrations of 5 to 20 μg/ml inhibited histamine release provoked by antigenic challenge of passively sensitized guinea-pig lung fragments (Figure 7).

Table 4 Inhibition of passive cutaneous anaphylaxis in the mouse induced by an IgE-like antibody

Compound	*Dose (mg/kg i.v.)*	*No. of animals*	*Mean score (± s.e. mean)*	*% inhib. (± s.e. mean)*
Control		42	8.3 ± 0.2	
DSCG	10	15	8.1 ± 0.6	3 ± 7.7
	20	15	8.2 ± 0.6	2 ± 7.9
	40	15	8.1 ± 0.3	2 ± 4.8
I.C.I. 74,917	0.5	18	6.1 ± 0.5	27 ± 6
	1.0	20	5.7 ± 0.6	32 ± 7
	2.5	23	2.8 ± 0.4	67 ± 5

Passive cutaneous anaphylaxis in the mouse

Unlike DSCG, I.C.I. 74,917 inhibited PCA in the mouse induced by the IgE-like antibody (Table 4). To achieve this effect, however, it was necessary to employ much higher doses than in the rat and guinea-pig. Inhibition, unlike that in the guinea-pig and rat (IgG-like and heterologous antibodies), was dose-dependent.

Discussion

I.C.I. 74,917 was similar to DSCG in its anti-allergic properties and it was in the rat that this similarity was most obvious. Both compounds inhibited PCA induced by the heat-labile homocytotropic antibody, but I.C.I. 74,917 was approximately 300 times more potent than DSCG in both the EA and *N. brasiliensis* systems (Table 1). To achieve maximal inhibition, it was necessary to administer either compound simultaneously with antigen. When given 10 min before the antigen, inhibition was reduced by some 70%; if given after, no inhibition was found (Figure 1, see also Cox, Beach, Blair, Clarke, King, Lee, Loveday, Moss, Orr, Ritchie & Sheard, 1970; Thomson & Evans, 1973). Neither compound inhibited the dermal reactions provoked by local injections of histamine and 5-HT, the two amines known to be responsible for the development of PCA reactions following interaction of IgE-like antibody and antigen (Mota, 1964). It is probable that in the rat, I.C.I. 74,917, like DSCG, prevents the release of inflammatory mediators from sensitized cells. This hypothesis was further supported by the findings that *in vitro*, both compounds when present at antigenic challenge reduced the amount of histamine released from peritoneal mast cells (Figure 2). A high degree of specificity was exhibited by each compound inasmuch as the most noticeable activity was found against responses provoked by IgE-like antibody. Only slight inhibition of reactions to IgG was demonstrated but it was significant, suggesting that specificity was not directed to a particular antibody-type, but more probably to a cell-type, perhaps the mast cell. The bulk of published evidence (summarized by Cox, 1971) would support this suggestion, and in view of the similarity in biological properties of these two compounds in the rat, it is possible that I.C.I. 74,917 acts in a similar manner.

The inhibitory effect on blueing reactions induced by Compound 48/80 was demonstrable only when DSCG or I.C.I. 74,917 was administered intradermally (Table 2). Neither compound was active when given intravenously, which conflicts with the finding of Orr, Hall, Gwilliam & Cox (1971) that DSCG at intravenous doses of 10 and 100 mg/kg inhibited the degranulation of mast cells induced by intradermal injections (0.125, 0.25 and 0.5 μg/site) of Compound 48/80. This discrepancy could possibly be related either to the different end-points employed, i.e. degranulation as opposed to blueing, or the different rat strains. Goose & Blair (1969), however, have reported that DSCG did not inhibit degranulation of mast cells provoked by Compound 48/80.

These studies, therefore, suggest that in the rat the basis of the anti-allergic activity of I.C.I. 74,917 may be the same as that of DSCG, that is, inhibition of mediator release from mast cells.

This similarity between the two compounds does not extend to other species. Thus in the mouse DSCG did not inhibit PCA reactions provoked by an IgE-like antibody (see also Cox *et al.*, 1970), whereas I.C.I. 74,917 did (Table 4). The latter compound may, as in the rat, inhibit the release of amines from murine mast cells, but direct evidence for this is not yet available. In the guinea-pig also, the pharmacological properties of DSCG and I.C.I. 74,917 differed markedly. Although recent reports (Assem, 1973; Taylor & Roitt, 1973) have shown that DSCG inhibits certain allergic reactions in the guinea-pig, no consistent or significant inhibitory activity was found in the present work at intravenous doses up to 50 mg/kg. In contrast, I.C.I. 74,917 was highly effective in inhibiting allergic bronchospasm provoked by a heat-stable antibody preparation (Figure 3). In preliminary studies the compound partially inhibited histamine release from passively sensitized chopped lung challenged with allergen, but the concentrations required *in vitro* (5 to 20 µg/ml) were high in comparison with those required *in vivo* (2.5 to 10 µg/kg i.v.). Furthermore, I.C.I. 74,917 had little effect upon PCA (Table 3) which could possibly be due to tissue specificity. The bronchospasm produced by I.C.I. 74,917 in the guinea-pig is not of reflex origin as it is demonstrable in an isolated lung preparation. It is unlikely to be related to the release of histamine, 5-HT or acetylcholine from lung tissue, as specific antagonists of these agents did not influence it. The role of other bronchoconstrictor substances, for example the kinins or prostaglandins, cannot be ruled out, but it is possible that the effect may be a direct one upon guinea-pig bronchial muscle. Further studies (unpublished data) have revealed no bronchospasm in the cat, dog, rabbit, rat, marmoset or monkey, or in normal human volunteers, following inhalation of I.C.I. 74,917. It would appear, therefore, that the effect is specific to the guinea-pig. While it is possible that the anti-allergic activity of I.C.I. 74,917 and the bronchospastic activity in guinea-pigs are related, more knowledge of the mode of action of this compound in both situations is required before this concept can be accepted.

In conclusion, therefore, these studies have revealed that I.C.I. 74,917 possesses some properties in common with DSCG, but acts in species and models in which DSCG is inactive. It is possible that I.C.I. 74,917 could represent an entirely different class of anti-allergic agent.

I.C.I. 74,917 was supplied by Mr W.S. Waring of our Chemistry Department and DSCG by Dr T.S.C. Orr of Fisons Pharmaceuticals Division. The expert technical assistance of Mrs G. Gorton, Mr P. Marshall, Mr C.P. Allott and Mr R.E. Anderson is gratefully acknowledged.

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EFFECTS OF APOMORPHINE, ERGOCORNINE AND PIRIBEDIL ON AUDIOGENIC SEIZURES IN DBA/2 MICE

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Audiogenic seizures in DBA/2 mice have been studied after administration of drugs believed to act as dopamine agonists Apomorphine at 0.4 mg/kg delays all phases of the response, the tonic phase is absent after 2.0 mg/kg; the clonic phase is abolished by 10 mg/kg. Ergocornine (0.5-8.0 mg/kg) produces effects on the latency and occurrence of seizure stages similar to those of apomorphine. Piribedil, ET 495 (4-100 mg/kg) is less potent; even after 100 mg/kg clonic and tonic phases occurred in 50% of the mice.

Introduction Certain inbred strains of mice, when 20-32 days old, show a sequence of epileptic-like activity (wild-running, clonic jerks, tonic flexion then extension, terminating with respiratory arrest) if subjected to a loud noise of mixed spectral composition (Collins, 1972). Several studies (Lehmann, 1964; 1967; 1970) implicate monoaminergic systems in the induction or evolution of these 'audiogenic seizures'. In particular, (+)-amphetamine (15 mg/kg) diminishes the incidence or severity of seizures. Reserpine enhances audogenic seizures; this effect is antagonized by L-3,4-dihydroxyphenylalanine (200 mg/kg) (Boggan & Seiden, 1971). We have therefore tested the effect on audiogenic seizures of three compounds believed on the basis of various biochemical and behavioural tests to act as dopaminergic agonists in the rodent brain.

Methods DBA/2 mice (Fisons Pharmaceuticals Ltd), 23-28 days old, weighing 7-13 g, were placed under a dome, diameter 58 cm, and habituated for 30 seconds. Stimulation by an electric bell (Friedland Chimes, 3 inch diameter, producing 109 dB at mouse level) was applied for 60 s or until tonic extension occurred. The appearance of seizure stages was timed. Mice were pretreated with intraperitoneal injection of 0.9% w/v NaCl solution (saline) or of solutions containing apomorphine hydrochloride (Evans Medical), ergocornine hydrogen maleinate (Sandoz Ltd) or piribedil (ET 495, 1-3,4-methylenedioxybenzyl-4-(2-pyrimidinyl) piperazine-methane sulphonate) (Servier Laboratories).

Results Spontaneous exploratory motor activity was observed during habituation after saline and all drug doses. Sniffing and stereotyped head movements were prominent after the highest doses of apomorphine, piribedil and ergocornine. The latter drug at 8 mg/kg sometimes evoked tremors and retropulsion. All animals were capable of running, but after the highest doses of apomorphine and ergocornine the limbs tended to be rigidly extended and the back arched.

After saline pretreatment 'wild running' was invariably observed during auditory stimulation; later seizure stages were seen in 80-100% of mice. Latencies to onset of the successive phases (mean in s ± standard error) were: wild running, 0.8 ± 0.1 ($n = 30$); clonic, 8.0 ± 1.3 ($n = 29$); tonic, 11.1 ± 1.4 ($n = 27$); and respiratory arrest, 22.5 ± 1.8 ($n = 26$).

Figure 1a shows the effect of apomorphine (0.08-10.0 mg/kg) on the occurrence of the different seizure stages. The tonic phase was abolished by apomorphine, 2.0 mg/kg, and the clonic phase was absent after 10 mg/kg. The latency of wild running was increased at all doses, reaching 3.5 ± 1.1 s ($n = 7$) at apomorphine, 2.0 mg/kg. Latency of the clonic phase was doubled after apomorphine, 0.08-2.0 mg/kg.

The occurrence of seizure responses after ergocornine is shown in Figure 1b. The wild running phase was markedly delayed after 8.0 mg/kg (mean latency 3.7 ± 2.3 s, $n = 8$). The tonic phase was abolished after 2.0 mg/kg; the clonic phase was delayed after 2.0 mg/kg, and absent after 8.0 mg/kg.

Piribedil increased the latency of the wild running phase (e.g. 2.3 ± 0.4 s, $n = 10$, after 20 mg/kg; 3.9 ± 0.7 s, $n = 8$, after 100 mg/kg). Doses in the range 4-100 mg/kg tended to reduce the incidence of later stages of the seizure response. However after 100 mg/kg, 70% still showed a clonic phase and 50% a tonic phase, but the extensor component of the tonic phase was seen in only 30% and respiratory arrest in only 10%.

Discussion McKenzie & Soroko (1972) demonstrated that apomorphine (2.5-10.0 mg/kg) protected rats against maximal electroshock seizures, but that mice were not protected by apomorphine (2.5-160.0 mg/kg). Apomorphine also suppressed firing in a cobalt-induced focus in the rat (Dow, Hill & McQueen, 1974). However, it potentiated pentylenetetrazol convulsions in both mice and

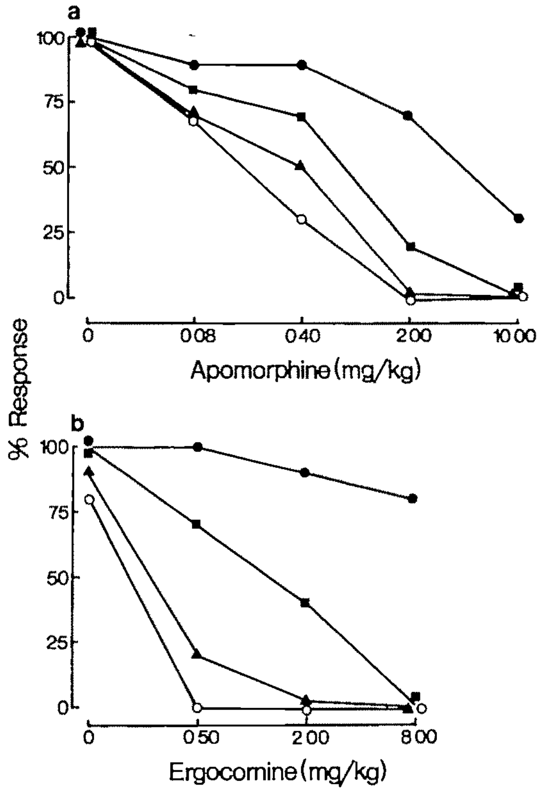

Figure 1 Graphs to indicate components of the seizure response to acoustic stimulation in DBA/2 mice. Five males and 5 females were tested after saline or each drug dose. The percentage showing the different seizure components is indicated as· (●) 'wild running', (■) clonic phase, (▲) tonic phase and (o) respiratory arrest. (a) Tests conducted 20 min after the intraperitoneal injection of saline or apomorphine hydrochloride (0.08-10.0 mg/kg). (b) Tests conducted 45 min after the intraperitoneal injection of ergocornine (0.5-8.0 mg/kg).

rats (Soroko & McKenzie, 1970). Our results demonstrate a powerful protective action of apomorphine (2.0-10.0 mg/kg) against audiogenic seizures in mice.

Evidence similar to that indicating a dopamine agonist action of apomorphine at receptor sites in the basal ganglia and elsewhere (Ernst, 1967; Anden, Rubenson, Fuxe & Hökfelt, 1967), has recently been produced for ergocornine (Fuxe, Corrodi, Hökfelt, Lidbrink & Ungerstedt, 1974), and for piribedil (Corrodi, Farnebo, Fuxe, Hamberger & Ungerstedt, 1972). However, the latter drug may owe its action on dopamine receptors to a metabolite (Miller & Iversen, 1974). Thus the strong anti-seizure effects of apomorphine and ergocornine (and the weaker effect of piribedil) and the similar effect of L-DOPA in reserpine-treated mice (Boggan & Seiden, 1971) may be a consequence of dopamine receptor activation. A role for dopaminergic systems in audiogenic seizures has yet to be defined, but there is evidence from several types of experiment that interaction between efferent and proprioceptive activity plays a part in the evolution of the seizure response.

The results with apomorphine, ergocornine and piribedil in DBA/2 mice are similar to those obtained in cobalt-induced epilepsy in the rat (Dow *et al.*, 1974). Thus the species difference between rats and mice observed for the anti-seizure effect of apomorphine (McKenzie & Soroko, 1972) appears to be a particular feature of tests using electroconvulsive shock. Although apomorphine and related drugs cannot be regarded as general anti-convulsants because of their lack of action against drug-induced seizures, a clear protective action has now been demonstrated in three very different rodent models of epilepsy.

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TETRAMETHYLENEDISULPHOTETRAMINE: AN INHIBITOR OF γ-AMINOBUTYRIC ACID INDUCED DEPOLARIZATION OF THE ISOLATED SUPERIOR CERVICAL GANGLION OF THE RAT

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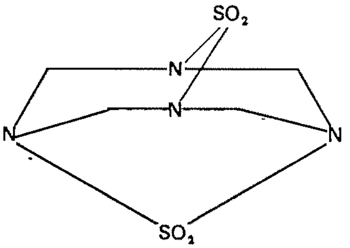
Tetramethylenedisulphotetramine (TETS), a potent convulsant, antagonized the depolarizing action of γ-aminobutyric acid (GABA) in the isolated superior cervical ganglion of the rat. No antagonism of responses to the cholinomimetic agent carbachol was observed. TETS appeared to act in a non-competitive manner and was reversible. Its activity profile was comparable to that of bicuculline in the same tissue except that the latter appears to act in a competitive manner.

Introduction It has recently been suggested that 2,6-dithia-1,3,5,7-tetraza-adamantane-2,2,6,6-tetraoxide [tetramethylenedisulphotetramine] (TETS) might be a γ-aminobutyric acid (GABA) antagonist (Smythies, 1974). In accordance with this speculation, TETS has convulsant activity (Hagen, 1950; Haskell & Voss, 1957, Voss, Haskell & Gartenberg, 1961) and has been stated to reduce possible GABA-mediated inhibition in the mammalian central nervous system (Curtis & Johnston, 1974).



Formula of tetramethylenedisulphotetramine (TETS)

In the present experiments we have assessed the antagonistic activity of TETS towards the depolarizing action of GABA on isolated sympathetic ganglia of the rat. This depolarization is mediated through receptors analogous to those in the central nervous system (Bowery & Brown, 1974) and the ganglion preparation is more suited for quantitative measurements since drugs can be applied in known concentrations.

Methods The procedure was essentially that described previously (Bowery & Brown, 1974) except that the moving-fluid electrode system was replaced by a vertical superfusion technique. Two Ag/AgCl electrodes were placed in electrical contact with the tissue, one via the thread attached to the post-ganglionic trunk and the other directly on the ganglion body close to the preganglionic trunk. The potential difference between these electrodes was monitored continuously on a Bryans 28000 potentiometric recorder. The tissue was superfused with Krebs solution at a rate of 1 ml min^{-1} and drugs were applied by addition to this solution.

Ganglionic uptake of tritiated GABA was determined as described previously (Bowery & Brown, 1972). Comparison was made between contralateral ganglia obtained from the same rats.

Synaptic transmission was monitored by suspension of the ganglion in a three-compartment bath (pre-, soma and post-, described in detail elsewhere) each chamber being perfused separately with Krebs solution at 0.6 ml min^{-1}. Stimulation (1 Hz, 1 ms) was applied to the preganglionic trunk through bipolar electrodes whilst recording from platinum electrodes placed in the soma and post-ganglionic trunk compartments.

TETS was prepared according to the method of Hecht & Heneka (1949) and its convulsant activity checked by intraperitoneal injection into mice: convulsions occurred at <0.25 mg kg^{-1}. Solutions containing TETS were obtained by dilution in Krebs solution from a freshly prepared stock of TETS dissolved in acetone (10 mg ml^{-1}). The apparent maximum concentration attainable was $<200\ \mu$M. In excess of this a precipitate rapidly formed. However, in one experiment a solution of 340 μM was used on the assumption that a maximum concentration was in solution. Acetone up to 0.5% in Krebs solution had no direct effect on the preparation or on the responses to GABA or carbachol.

(+)-Bicuculline-methochloride (methyl bicuculline chloride) was prepared by a modification of the method of Johnston, Beart, Curtis, Game, McCulloch & McLachlan (1972).

Results TETS antagonized the depolarizing action of GABA at concentrations of 10 μM upwards. Antagonism was fully reversible on washing. The effect appeared to be non-competitive; the log dose-response curve was shifted to the right and the maximum depolarization was diminished (Figure 1a). In contrast, methylbicuculline and bicuculline produced a parallel shift without depression, as previously noted for bicuculline alone (Figure 1b; see also Bowery & Brown, 1974).

TETS did not significantly inhibit the depolarization produced by carbachol at concentrations in excess of 130 μM (Figure 1c) and did not affect transmission of orthodromic stimuli. GABA depresses orthodromic transmission (de Groat, 1970; Adams & Brown, 1973). This depression appeared resistant to both TETS and bicuculline. The lack of effect by bicuculline confirms results previously obtained by de Groat, Lalley & Block (1971).

Glial cells in sympathetic ganglia accumulate GABA by a high-affinity carrier-mediated process (Bowery & Brown, 1972; Young, Brown, Kelly & Schon, 1973). Accumulation of GABA following incubation for 30 min in 0.1 μM [^{3}H]-GABA was not reduced by 130 μM TETS (added 30 min before GABA). Ganglionic bath fluid concentration ratios were: minus TETS (control) 9.82 ± 0.69; plus TETS (test) 11.61 ± 1.10 (mean ± s.e.mean, $n = 4$ in each case).

Discussion The results of these experiments would accord with Smythies' (1974) prediction that TETS blocks GABA receptors, in that it preferentially antagonized GABA-depolarization as against carbachol depolarization. Further, a preliminary test (Adams & Brown, unpublished) has indicated that TETS does not itself alter the resting potential or membrane conductance of ganglion cells.

The potency of TETS as a GABA-antagonist is not dissimilar to that of bicuculline or methyl bicuculline. However, the nature of the antagonism appears to be rather different, in that TETS reduced the maximum depolarization. This was not indicative of irreversible inhibition: effects of both TETS and bicuculline were rapidly reversed on washing. One possibility is that TETS occludes the GABA-ionophore in addition to, or instead of blocking receptors.

Previous experiments (de Groat, 1970; Bowery & Brown, 1974) have demonstrated an essential identity between GABA-receptors at the ganglion

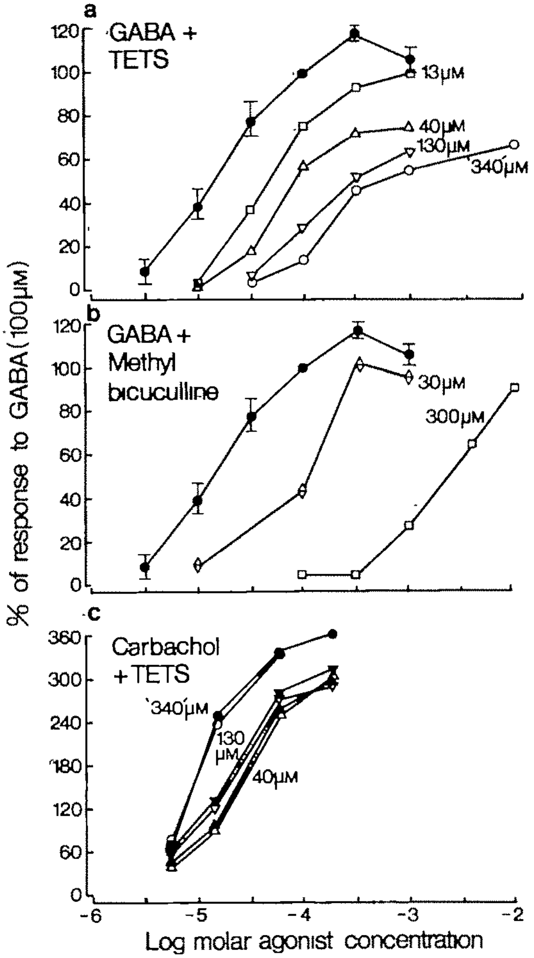


Figure 1 The effects of tetramethylenedisulphotetramine (TETS) (a & c) and methyl bicuculline (b) on ganglionic depolarization produced by γ-aminobutyric acid (GABA) (a & b) and carbachol (c). Ordinates in each case· peak depolarizations produced during 1 min applications of either GABA or carbachol, plotted as a percentage of the response to 100 μM GABA in that preparation (100 μM GABA = 100%) Abscissae log molar concentrations of GABA (a & b) or carbachol (c). Solid symbols represent the control responses to either GABA or carbachol; open symbols the responses obtained in the presence of different concentrations (μM) of TETS or methyl bicuculline as indicated on the figure The mean control log-dose response curve to GABA (a & b) was calculated from results obtained in 6 separate ganglia. Vertical bars represent s.e.mean. GABA or carbachol was applied at not less than 15 min intervals. TETS or methyl bicuculline was in contact with the tissue at least 20 min before application of the agonists. One concentration of TETS or methyl bicuculline was examined on a single ganglion. Note that '340' μM refers to an incomplete solution of TETS (see methods section).

and in the mammalian central nervous system with respect to ligand specificities. Consequently, it seems highly probable that TETS is capable of antagonizing actions of GABA elsewhere in the mammalian nervous system. However, it should be emphasized that ganglia are not sensitive to glycine or glutamic acid so we cannot determine in this preparation whether the effects of amino acids acting on other receptors are sensitive to TETS. Preliminary observations by Dray (1975) suggest that intravenous TETS may block the effects of iontophoretic glycine and GABA in the rat mid brain.

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CHANGES OF HUMAN PLASMA DOPAMINE-β-HYDROXYLASE ACTIVITY AFTER INTRAVENOUS ADMINISTRATION OF THEOPHYLLINE

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The intravenous administration of theophylline to ten healthy human subjects produced either an increase of circulating plasma dopamine-β-hydroxylase or no change. The rise of plasma enzyme activity may reflect the increased peripheral catecholamine release induced by theophylline.

Introduction Dopamine-β-hydroxylase (3,4-dihydroxyphenylethylamine, ascorbate oxygen: oxireductase (beta-hydroxylating) E.C.1.14.17.1) is localized in the chromaffin granules of the adrenal medulla (Kirshner, 1957) and in the synaptic vesicles of the nerve endings (Potter & Axelrod, 1963). It has been suggested that exocytosis is the mechanism by which noradrenaline secretion occurs from the adrenal medulla (Viveros, Arqueros & Kirshner, 1968) and the sympathetic nerves (Geffen, Livett & Rush, 1969; Weinshilboum, Thoa, Johnson, Kopin & Axelrod, 1971). Dopamine-β-hydroxylase (DBH) activity has been demonstrated in the blood of man and other species (Weinshilboum & Axelrod, 1971) and it has been proposed that plasma DBH activity may serve as an index of the activity of the sympathetic nervous system (for review, see Geffen, 1974).

Methylxanthines have been reported to cause release of catecholamines from the adrenal medulla (Peach, 1972) and sympathetic nerves (Wooten, Thoa, Kopin & Axelrod, 1973). The present investigation was done with the aim of supporting the hypothesis that DBH activity in the serum may serve as a reliable index of catecholamine release in man as judged by variations of the enzyme activity following the intravenous injection of theophylline.

Methods Theophylline (200 mg) was administered intravenously to 10 healthy friends or employees of the Neurological Clinic of the Hospices Civils (Strasbourg). At the indicated time intervals, a sample of blood was drawn from an antecubital vein for the measurement of DBH activity. The blood was collected in heparinized tubes, placed on ice and centrifuged. The plasma was frozen at −30° C until assayed. Plasma DBH activity was estimated according to the technique of Nagatsu & Udenfriend (1972), except that Cu^{2+} was added to the incubation mixture instead of N-ethylmaleimide to reverse endogenous inhibitors. For each individual sample, DBH activity was determined without copper (non-stimulated DBH activity) and at the optimal copper concentration which gave maximal stimulation (copper-stimulated DBH activity). All DBH assays were performed in duplicate on separate 50μl aliquots of plasma and the results of the two measurements were found to fall within ±0.5 units of the mean of the two analyses. The DBH activity was expressed as μmol of octopamine formed per min and per litre of plasma (International Units, i.u.).

Results The data presented in Figure 1 illustrate the changes in circulatory DBH activity following theophylline administration to various individuals. Whereas no change could be observed in five subjects, theophylline induced an increase of DBH activity in the other five. The maximal increase of the non-stimulated DBH activity was seen 1 h after the injection and reached from 15 to 125% of the initial DBH level, depending on the individual. On the other hand, the copper-stimulated DBH activity only increased from 23 to 36% of the initial value and there was a tendency to a further increase between 1 and 2 hours. For each individual, the rise of DBH activity when expressed in number of units was found to be the same in both copper-stimulated and non-stimulated DBH activities.

Discussion Our results show that theophylline induces an increase of DBH levels with a maximum occurring 1 h after the administration. The increase, however, was only observed in 50% of the subjects tested. It should be noted that Snider & Waldeck (1974) have shown that relatively high concentrations of aminophylline are required to induce the release of catecholamines from the isolated adrenal gland. Moreover, it should be emphasized that considerable variation of

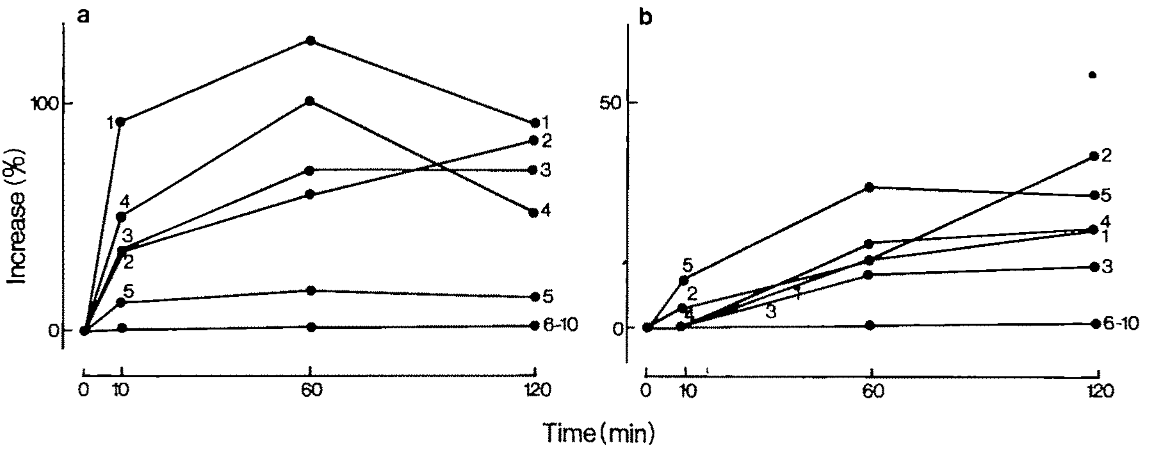

Figure 1 Percent increase in non-stimulated (a) and copper-stimulated (b) dopamine-β-hydroxylase activity after theophylline administration to ten human subjects (age range: 19-70, mean· 37). Values are recorded as percent change from initial values Numbers on the figure refer to individual subjects. Samples of blood were taken at 10, 60 and 120 min after theophylline administration. The mean value of initial DBH activity of the ten patients was 36 i.u. ± s.e.mean 5, (range. 20-58), for the copper-stimulated activity and 11 I.u. ± s.e.mean 2, (range· 3-17), for the non-stimulated activity. In separate experiments, theophylline was demonstrated to have no activating or inhibiting effect upon DBH.

individual plasma DBH levels can occur (Freedman, Ohuchi, Goldstein, Axelrod, Fish & Dancis, 1972; Wetterberg, Aberg, Ross & Fröden, 1972; Horwitz, Alexander, Lovenberg & Keiser, 1973; Miras-Portugal, Aunis, Mandel, Warter, Coquillat & Kurtz, 1975). Thus it is possible that the theophylline susceptibility differs from one individual to another and that the dose used in our experiments (which was limited by the toxic effect of higher doses of theophylline) was not sufficient to induce DBH release in some subjects.

Variations of DBH activity are determined by changes in its rate of release but also by changes in its rate of clearance from plasma and presumably by the action of endogenous inhibitors (not all of them being reversible by copper, e.g. plasma proteinases). The rise of DBH activity lasted for 1 h or longer. After 1 h, the haemodynamic changes provoked by the theophylline are no longer observable. Therefore, plasma volume changes which might cause variations of plasma DBH activity over a short time interval (Stone, Kirshner, Gunnells & Robinson, 1974; Miras-Portugal *et al.*, 1975) are presumably not responsible for the effect we observed. Thus the rise of plasma DBH activity described in this paper may reflect accurately the increased peripheral catecholamine release induced by theophylline.

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SENSITIVITY OF NEONATAL RABBIT ILEUM TO HISTAMINE

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Segments of ileum from newborn rabbits contracted in response to histamine but sensitivity declined from age 11 days. Intestine from adult animals barely contracted to histamine, even in a concentration of 3×10^{-3} M. Such variation with age was not observed with acetylcholine. The response to histamine was effected by mepyramine-sensitive receptors which appeared to decrease in number as the animals aged.

Introduction The intestine of the adult rabbit is notably insensitive to histamine compared with that of other species. During an investigation into the effects of field stimulation on the isolated intestine of neonatal rabbit, it was noticed that such preparations contracted maximally to concentrations of histamine that caused no reaction from adult rabbit intestine.

Further investigation showed that the sensitivity to histamine declined from age 11 days. Intestine from adult animals barely contracted in response to histamine even in a concentration of 3×10^{-3} M. Such variation with age was not observed with acetylcholine.

Methods Eighty-five preparations from 50 male and female rabbits were used. Strains used were Old English, New Zealand White, Belgian hare, Flemish giant, Swiss hare-type rabbits and a cross between Old English and New Zealand White. Segments of terminal ileum (3 cm long) were suspended in a modified Krebs fluid (Botting & Salzmann, 1974) gassed with 95% O_2 and 5% CO_2 at 34° C. Six cm lengths of ileum from neonatal animals were sometimes looped double to provide a more robust preparation. Contractions were recorded isotonically with a frontal writing lever or transducers (load 1 gram).

Intact, finely cut or homogenized intestine from adult or neonatal animals was incubated at 37° C with solutions of histamine (10^{-6} and 3×10^{-5} M) in Krebs solution. Histamine content of the incubate at various times up to 2 h was estimated on isolated segments of guinea-pig ileum suspended in Krebs solution. The effects of inhibitors of imidazole-*N*-methyl transferase (chlorpromazine, cupric chloride) and of diamine oxidase (aminoguanidine, semicarbazide, iproniazid) on the contractions produced by high concentrations of histamine were investigated on intestine from adult animals. The effects of indomethacin, hyoscine and tetrodotoxin (Sankyo Co. Ltd., Japan) on the histamine-induced contractions of neonatal ileum were investigated The pA_2 value of mepyramine against histamine on neonatal ileum was estimated by the method of Lockett & Bartlet (1956).

Results Neonatal rabbit ileum (6-20 days) contracted in response to histamine in concentrations of 3×10^{-7} M to 2.5×10^{-5} M. This was observed with all strains of rabbit used except New Zealand Whites, which were no more sensitive than adult animals, although when New Zealand Whites were crossed with Old English the ileum of all of the progeny contracted with histamine. The sensitivity developed at about 12 days of age. Contractions produced were equivalent to those produced by acetylcholine (10^{-6} to 10^{-5} M) although sometimes the maximum contraction to histamine was 20-30% greater than the maximum response induced by acetylcholine. Sensitivity to histamine declined with age, and in the adult rabbit the maximum response obtainable even with a very high concentration of histamine (3×10^{-3} M) was never greater than 25% of the maximum contraction produced by acetylcholine. Figure 1 illustrates this change of histamine sensitivity with time in five litter mates of different age. Sensitivity of the ileum to acetylcholine increased slightly with the age of the animal, although the slope of the dose-response curves and height of maximum contraction were approximately the same. In neonates the responses to histamine were unaffected by hyoscine (4×10^{-7} M), tetrodotoxin (3×10^{-6} M) and indomethacin (2.8×10^{-5} M), but were inhibited by mepyramine. A single estimate of the pA_2 at 2 min of mepyramine against histamine gave a value of 8.9. The small contractions produced by high concentrations of histamine applied to the ileum of adult rabbits were not affected by aminoguanidine (7×10^{-5} M), semicarbazide (9×10^{-6} M), iproniazid (1.5×10^{-6} M) and

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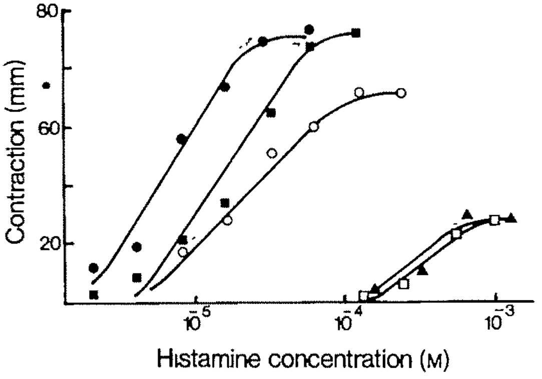

Figure 1 The effect of histamine on terminal ileum of five litter-mate rabbits, age. (●) 12 days, (■) 6 days, (○) 14 days, (▲) 37 days and (□) 60 days. Each point is the mean of two contractions of a·3 cm length of tissue recorded isotonically with a load of 1 g and a magnification of 7.

chlorpromazine (3×10^{-6} M). Copper chloride (1.7×10^{-5} M) caused about 10% potentiation of the histamine response.

Breakdown of histamine by intact, minced or homogenized intestine was slightly more rapid with neonatal ileum than with adult ileum.

Discussion The sensitivity of neonatal rabbit ileum to histamine, compared to the adult, is not due to more effective catabolism in older animals, since the breakdown of histamine incubated with intestine is not greater with preparations from adult animals, and inhibition of enzymes responsible for histamine breakdown failed to potentiate histamine responses in the ileum of mature animals. Apart from these observations, if increased metabolism were the reason for the insensitivity of the intestine of adult rabbits one would expect this tissue to give dose-effect curves of similar slopes and maxima to those of neonatal tissue, when much greater concentrations of histamine were used. Neither is the action in neonatal animals likely to be attributable to an indirect action of histamine, since it is unaffected by an inhibitor of nerve activity, an inhibitor of prostaglandin synthetase or a muscarinic receptor blocking drug. The similarity of the pA_2 (8.9) with that reported by Schild (1947) for histamine against mepyramine on guinea-pig ileum (8.7) suggests that the action is effected by stimulation of H_1 histamine receptors. Presumably neonatal rabbit intestine possesses large numbers of H_1-receptors, which are either lost or rendered ineffective in adult animals.

Field stimulation of ileum from neonatal rabbits, compared with similar experiments on tissue from adults indicate that the intramural nerves are not fully functional in the rabbit until about 12-20 days of age (unpublished observations). Similar observations were reported by Burn (1968) with respect to the function of adrenergic nerves. Experiments are in progress to investigate the effect which chronic denervation of adult rabbit intestine may have on histamine sensitivity.

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COMMUNICATIONS

In communications with more than one author, an asterisk (*) denotes the one who presented the work.

Pharmacological effects of histamine on the isolated cat nictitating membrane

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Histamine is a direct stimulant of the smooth muscle in several tissues. Yet, using isotonic recording Thompson (1958) reported that low concentrations of histamine in only a few preparations stimulated the smooth muscle of the cat nictitating membrane.

In the present studies, carried out with the isolated medial muscle of the cat nictitating membrane, it was found that histamine stimulated this smooth muscle and the effect was concentration-dependent (5.4 μM to 5.4 mM). The maximum development of tension in response to histamine was 13.2 ± 0.6 g in the controls ($n = 8$). The maximum tension developed by (–)-noradrenaline under these experimental conditions was 19.2 ± 1.6 g ($n = 9$). The effect of histamine does not seem to be mediated through the activation of alpha-adrenoceptors because the concentration-effect curve for histamine was not affected in the presence of 2.9 μM phentolamine. However, the responses to the highest concentration of histamine employed (5.4 mM) appear to be related to the release of endogenous noradrenaline because they were reduced after pretreatment with reserpine (0.3 mg/kg, 24 h before the experiment).

The responses to exogenous (–)-noradrenaline were not affected in the presence of either 5.4 or 16 μM histamine (pD_2 in the controls: 5.04 ± 0.04; histamine 5.4 μM: 5.08 ± 0.03 and histamine 16 μM: 5.12 ± 0.04).

In the tissues previously labelled with ^{3}H-noradrenaline, exposure to 16 or 54 μM histamine produced a concentration-dependent increase in the spontaneous outflow of radioactivity: 1.65 ± 0.09-fold with 16 μM ($n = 8$) and 3.36 ± 0.21-fold with 54 μM histamine ($n = 4$). Under these experimental conditions the deaminated glycol, ^{3}H-DOPEG (3,4-dihydroxyphenylglycol) accounted for more than 75% of the total increase in outflow of radioactivity elicited by exposure to histamine. In the presence of pirylamine 2.7 μM the releasing effect of histamine, 54 μM, was markedly reduced.

Transmitter overflow elicited by postganglionic nerve stimulation (10 Hz, during 2 min, supramaximal voltage) was increased approximately 2-fold in the presence of 16 or 54 μM histamine The fraction of the tritiated noradrenaline released by nerve stimulation which was collected as ^{3}H-DOPEG was increased significantly in the presence of histamine. Simultaneously, there was a marked reduction in the formation of ^{3}H-normetanephrine from ^{3}H-noradrenaline released by nerve stimulation.

The effects of histamine on adrenergic nerve endings are considered of interest because of the known effects of this amine on the cell body of the postganglionic adrenergic neurone. In the superior cervical ganglion, histamine has a facilitatory effect on neurotransmission which is mediated by H_1 receptors, and an inhibitory effect which is mediated by H_2 receptors (Brimble & Wallis, 1973). Consequently, the effects of histamine on adrenergic neurotransmission and on the smooth muscle of the nictitating membrane were studied in the presence of agents which block either H_1-receptors (pirylamine) or H_2-receptors (burimamide).

It is concluded that histamine has a prejunctional effect which resembles the effects of reserpine-like agents (Adler-Graschinsky, Langer & Rubio, 1972; Graefe, Stefano & Langer, 1973). In addition, histamine enhances ^{3}H-noradrenaline overflow elicited by nerve stimulation and it stimulates the smooth muscle of the cat nictitating membrane.

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Dose response relationship and comparison of the secretory potency of methyl histamine and histamine on the isolated guinea pig stomach

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The side-chain-methylated derivatives Nα-methyl histamine (NαMeH) and Nα, Nα-dimethyl histamine (NαMe$_2$H) are more active than histamine in stimulating gastric secretion (Code, Maslinski & Mossine, 1971). Code (1973) has therefore suggested that these Nα-methyl histamines may be physiological stimulators of parietal cells. In the guinea pig NαMeH and NαMe$_2$H were less potent than histamine in stimulating H_1-receptors to produce broncho-constriction, increased permeability of skin vessels and contraction of the isolated ileum (Bertaccini & Vitali, 1964). 5-methyl histamine (5MeH) is a gastric secretagogue of approximately equal potency with histamine in various species but has relatively little activity on H_1 receptors (Bertaccini & Impicciatore, 1974; Black, Duncan, Durant, Ganellin & Parsons, 1972). We have now compared the potency of these three histamine derivatives with histamine on the H_2-receptors of the guinea pig stomach.

Male guinea pigs (300-500 g) were given a low residue diet with water freely available for 48 h before the experiment. The guinea pig was killed by a blow on the head followed by bleeding. Half-stomach preparations were set up as described previously (Spencer, 1973). 4 or 5 dose levels, differing by a factor of two, of the four test substances were randomized between 168 preparations. After a base-line secretory plateau to theophylline hydrate (0.2 mg/ml) had been obtained, the test substance was administered to the serosal side for 60-90 min and then removed by washing the preparation twice. The base-line secretion level was regained before applying another dose. Usually three or four tests could be completed on each preparation. The rate of acid secretion (μEq.cm^{-1}h^{-1}) stimulated by each dose was calculated as the difference between the mean of 3 consecutive periods of test stimulation 30 min after administration of the test and the mean of 3 consecutive periods of theophylline-induced secretion immediately before administration of the drug.

The highest dose of each drug gave a smaller mean response than the second highest dose indicating that the maximum response had been obtained. Omitting the highest dose tested, the mean response for each drug was linearly related to the log dose. The common regression coefficient

$$(b) = \frac{\text{response}}{\log_{10}\text{dose}},$$

was obtained by the method of Snedecor & Cochrane (1969). b = 2.06, (fiducial limits 1.20, 2.92 at $P = 0.05$). None of the regression coefficients for the four drugs differed significantly from the common regression coefficient. The molar potency ratios with reference to histamine were NαMeH 2.3 ± 0.22 s.e. mean NαMe$_2$H, 0.85 ± 0.26 s.e. mean, 5MeH 0.43 ± 0.27 s.e. mean.

We have therefore confirmed that NαMeH is a more potent stimulus for gastric acid secretion than histamine but the potency ratio in the guinea pig is less than that observed in cats and dogs.

We have also shown that in the guinea pig NαMe$_2$H is an approximately equipotent and 5MeH is a less potent H$_2$-receptor agonist than histamine.

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Effects of atropine on gastric secretion and mucosal blood flow in conscious dogs.

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Atropine is known to decrease gastric acid secretion in the dog (Gregory & Tracy, 1961) rat (Johansson, Lundell & Svensson, 1971) and cat (Svensson & Emas, 1974), and is still used clinically as an anti-secretory and spasmolytic drug. For therapeutic use an anti-secretory drug is likely to be more beneficial if it does not greatly reduce the ratio of gastric mucosal blood flow (MBF) to secretory rate.

We have examined the effects of atropine on MBF and acid secretion in six dogs with Heidenhain pouches. Food was withheld but water allowed for 18 h before experiments. Submaximal gastric acid secretion was induced by pentagastrin (1-4 μg kg^{-1} h^{-1}) or bethanechol (60 μg kg^{-1} h^{-1}). Atropine was given during the secretory plateau. Secretion was collected over 15 min periods and acid output measured by titration. MBF was estimated by radioactive aniline clearance (Curwain & Holton, 1973). Effects on acid secretion and MBF were calculated as follows: Measurements for the three 15 min periods preceding the dose of atropine were compared with the mean of the three values from 15-60 min after atropine. Results are expressed as percentage changes ± s.e. mean.

In four experiments in four dogs atropine sulphate (100 μg/kg i.v.) decreased pentagastrin-stimulated acid secretory rate (62.8 ± 12.4) and MBF (44.5 ± 18). The concentration of acid secreted decreased by 14.8 ± 3.7 and 23.5 ± 7.5 respectively 45 and 60 min after atropine, but the pH of the secretion was less than 3.0. The ratio of MBF to secretion rose (39.3 ± 12.8) during secretory inhibition.

Atropine sulphate (100 μg/kg i.v.) also decreased acid secretion stimulated by bethanechol. In three experiments in three dogs secretion decreased by a mean of 83.0 ± 11.0. In one of these experiments the pH of the secretion remained less than 3.0 and the ratio of MBF to secretion rose by 56% during secretory inhibition. In the other two experiments acid concentration fell so much that the pH was above 3.0. Under these conditions aniline clearance does not measure MBF.

In twelve further experiments smaller doses of atropine (1-25 μg/kg i.v. or s.c.) inhibited secretion in response to both stimuli but were more effective against bethanechol. In the seven experiments in which the pH of the secretion remained below 3.0 the ratio of MBF to secretory rate rose (55.0 ± 16 6). We conclude that atropine does not inhibit acid secretion by decreasing MBF.

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The effect of atropine on pentagastrin-induced gastric acid secretion and mucosal blood flow in the conscious rat

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Atropine is known to decrease gastric acid secretion in response to a variety of stimuli (Hirschowitz & Sachs, 1969; Johansson, Lundell & Svensson, 1971). In the present series of experiments the effect of atropine 100 μg/kg i.v. was investigated for its effect on pentagastrin-induced gastric acid secretion and gastric mucosal blood flow in the conscious rat.

Male and female Wistar rats were provided with either vagally innervated (Pavlov) pouches or vagally dennervated (Heidenhain) pouches using established surgical procedures (Svensson, 1970). Three weeks later indwelling vascular catheters (Weeks, personal communication) were implanted. Drugs were dissolved in sterile pyrogen-free saline and administered via the jugular catheter. Acid secretion was collected by pouch perfusion and measured every 15 min by titration to pH 7.4 using an automatic titrator. The results were expressed in μEq H$^+$ min^{-1}. Blood samples (0.01 ml) were withdrawn via a catheter in the carotid artery. Mucosal blood flow (MBF) in ml min^{-1} was estimated using the radioactive aniline clearance method developed for the conscious dog (Curwain & Holton, 1973) and adapted for use in the conscious rat. 18 h before each experiment food was withdrawn but water and Tyrode's solution allowed. Pentagastrin dose-response curves for each rat were determined and the dose which produced secretion at about 50% maximum was used for the rest of the study. A steady plateau of acid secretion and MBF was first obtained using pentagastrin. The ratio of mucosal blood flow to acid secretion was steady at 0.09 + 0.011 ml (μEqH$^+$)$^{-1}$. The effect of atropine was calculated from the mean secretory rate and MBF of the three samples at 30, 45 and 60 min after atropine compared with the three samples immediately before giving atropine. The results are expressed as percentages s.e. mean.

In eleven experiments on three rats with Pavlov pouches and three rats with Heidenhain pouches atropine caused a 75.8 ± 2.95% fall in acid secretion with only a 39.8 ± 3.17% fall in MBF. No significant difference was found between the extent of inhibition in the two types of pouches. Acid secretion and MBF after atropine were significantly different from their pre-atropine levels. The percent decrease in MBF was also significantly different from the percent decrease in acid secretion.

An increase in the ratio of MBF to secretory rate was always seen, indicating a primary effect on the secretory apparatus and not blood flow.

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R.L. McIsaac holds a post-graduate studentship from St. Mary's Hospital Medical School.

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The pharmacology of cimetidine, a new histamine H_2-receptor antagonist

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Burimamide and metiamide which have been described previously (Black, Duncan, Durant, Ganellin & Parsons, 1972; Black, Duncan, Emmett, Ganellin, Hesselbo, Parsons & Wyllie, 1973) are histamine H_2-receptor antagonists This communication describes some aspects of the pharmacology of cimetidine (N-cyano-N′-methyl-N″[2-(5-methyl-4-imidazolyl-methylthio)ethyl] guanidine; SK&F 92334), a new H_2-receptor antagonist. *In vitro* the compound antagonizes the actions of histamine on isolated guinea-pig atrium and isolated electrically-stimulated rat uterus with K_B values of 7.9×10^{-7}M and 8.1×10^{-7}M respectively, corresponding to pA_2 values of 6.1 on each tissue. At very high concentrations cimetidine antagonizes the actions of isoprenaline on atrium and uterus and the actions of histamine and carbachol on isolated guinea-pig ileum but the results are not consistent with competitive antagonism at β-adrenoceptors, histamine H_1-receptors or muscarinic receptors.

The effects of cimetidine on gastric acid secretion have been studied in a number of preparations. The results are summarized in Table 1. In all preparations cimetidine was approximately equiactive in inhibiting histamine- and pentagastrin-stimulated acid secretion but less effective in inhibiting carbachol-stimulated secretion. Basal secretion was also inhibited In Heidenhain pouch dogs the blood levels to give 50% inhibition of maximally-stimulated gastric secretion (EC50) were approximately 1-2 μM and the half-life of the compound about one hour.

In male human volunteers cimetidine given intravenously has been shown to inhibit histamine- or pentagastrin-stimulated gastric secretion with an EC50 of about 2.5 μM and a half-life of about two hours.

TABLE 1 The effects of cimetidine on gastric acid secretion

Preparation	*Stimulant*	*Effect of Cimetidine*
Rat: Lumen-perfused stomach	Histamine 15 μmol kg^{-1} h^{-1}	ID50 (rapid i.v. injection) 1.37 μmol/kg ID50 (intraduodenal administration) 5.5 μmol/kg i.v. infusion of 3 μmol kg^{-1} h^{-1} produced mean inhibition of 71%
	Pentagastrin 60 μg kg^{-1} h^{-1}	ID 50 (rapid i.v. injection) 1.4 μmol/kg
	Carbachol 30 μg kg^{-1} h^{-1}	Variable effect. Significant inhibition at 8 μmol/kg Approximately 50% inhibition at 128-256 μmol/kg
Rat. Gastric fistula	Basal secretion	i.v. infusion of 6 μmol kg^{-1} h^{-1} produced mean inhibition of 20% in first hour and 30% in second hour. With 60 μmol kg^{-1} h^{-1} inhibitions were 71% and 96% respectively.
Cat: Lumen-perfused stomach	Histamine 3 μmol kg^{-1} h^{-1} Pentagastrin 10 μg kg^{-1} h^{-1}	ID50 (rapid i.v. injection) 0.85 μmol/kg ID50 (rapid i.v. injection) 1.46 μmol/kg
Dog: Heidenhain pouch	Histamine 1.3 μmol kg^{-1} h^{-1}	ID50 (rapid i.v. injection) 1.7 μmol/kg ID50 (i.v. infusion) 4.7 μmol kg^{-1} h^{-1} Oral administration of 10 & 20 μmol/kg produced mean inhibitions of 70 & 90% respectively
Dog: Heidenhain pouch	Pentagrastrin 8 μg kg^{-1} h^{-1}	2 μmol/kg by rapid i.v. injection gave mean inhibition of 55%
	Carbachol 6.7 μg kg^{-1} h^{-1}	4 μmol/kg by rapid i.v. injection gave mean inhibition of 59%

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In chronic toxicity studies metiamide has been shown at high doses to produce kidney damage and agranulocytosis in some dogs (Brimblecombe, Duncan & Walker, 1973). In tests so far carried out cimetidine at equivalent doses has not shown similar toxicity.

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Observations on the effect of dibenzoxazepine (CR) and N-nonoyl-vanillylamide (VAN) on sensory nerves

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VAN and CR cause a burning pain when applied to the human skin (Ballantyne, Beswick & Price-Thomas, 1973). This action has been investigated in cats by the application to a burn blister and to intact skin on the hindleg. Recordings were made from the sensory nerves innervating these areas.

Cats were anaesthetized with α-chloralose 70-90 mg/kg, and the blood pressure was monitored. The saphenous nerve was exposed in the thigh. The nerve was immersed in liquid paraffin and prepared for multifibre and single unit recording (Iggo, 1960). Nerve activity was recorded by bipolar silver electrodes, displayed on an oscilloscope for photography and stored on magnetic tape for further analysis using a small computer. Sensory units were identified by their response to mechanical stimulation of the skin, and by the conduction velocity and/or duration of the action potential in the related nerve fibres. Burn blisters were prepared by placing a metal disc (2 cm) at 100°C on the skin for 10 seconds. Close-i.a. injection of drugs employed the saphenous artery.

Only in four out of ten experiments where a blister had been prepared were the nerves innervating the blister identified. Isotonic KCl was applied in all four experiments to the blister base and found to cause activity in the isolated nerve fibres. Onset of action varied between 5 s and 1 min 45 seconds. On application of CR or VAN 10^{-4} M in saline no effects attributed to the drugs were observed. To test whether the preparations had been desensitized isotonic KCl was added again and was found to be effective in all cases.

Application of CR 10^{-4} M to intact skin. In six out of twenty-one preparations C-fibre units were isolated. These fine strands of nerves also contained in most cases alpha mechanoreceptors which were of low threshold and failed to respond. Five of these C-fibre units responded to CR and comprised 2 moderate threshold mechnoreceptors, 1 high threshold mechanoreceptor, 1 thermoreceptor and 1 unidentified receptor unit. Onset of activity in these units ranged between 5-8 min except for high threshold unit where the skin area was broken and activity occurred in 5 seconds. All these units showed immediate tachyphylaxis but still responded to physical stimulation. In some of these units, especially the thermoreceptor, saline was applied to the skin 45 min after CR response had subsided. Activity occurred within 5 min 5 seconds. This could be related to a similar phenomenon observed on human skin (Ballantyne, Beswick & Price-Thomas, 1973). All fibres affected by CR responded to 30 μg 5-HT and to 30 μg bradykinin injected close-i.a. Both compounds showed tachyphylaxis, but 5-HT more readily. Only some α fibres were affected by these compounds, probably the slowly adapting units (Fjallbrant & Iggo, 1961; Beck & Handwerker, 1974).

The observations suggest that CR acts on specific sensory units related to unmyelinated fibres. Research is continuing to clarify if the effect is indirect or direct and rigidly classify the fibre units which these compounds excite.

We wish to thank Professor A. Iggo for very helpful discussions and for demonstrating the technique.

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Actions of glutamate and kainic acid on the lobster muscle fibre and the frog spinal cord

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As kainic acid (Kai) is a potent glutamate agonist in the mammalian central nervous system (Shinozaki & Konishi, 1970, Johnston, Curtis, Davies & McCulloch, 1974), we have investigated the effect of Kai on two non-mammalian preparations where glutamate is a putative excitatory transmitter (Johnson, 1972). (1) Intracellular recordings were made from single muscle fibres in the lobster walking leg and the effects of Kai and of glutamate were measured as membrane depolarizations. (2) The frog isolated spinal cord preparation was studied according to the method of Mitchell & Phillis (1962) and the spinal acetylcholine (ACh) output was bioassayed every 10 minutes.

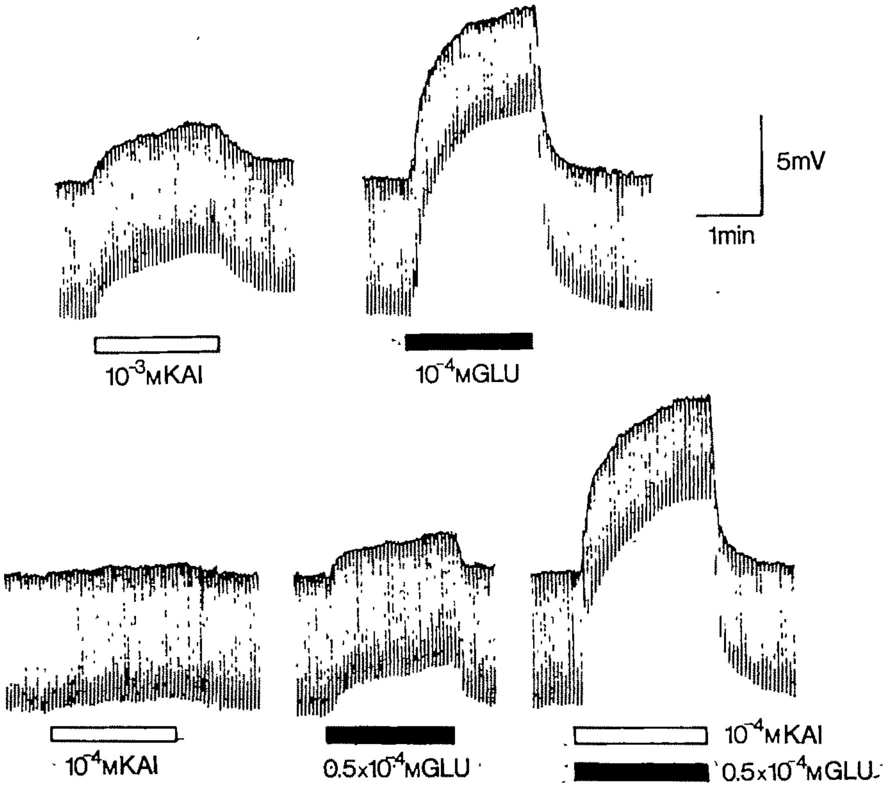

Figure 1 Depolarizations (upward deflections) induced by L-glutamate (GLU: solid bars) and kainic acid (KAI: open bars) in a single lobster muscle fibre. One voltage recording and one current (1.3×10^{-7} A) passing microelectrode were inserted into the middle of the fibre; the resultant electronic potentials were recorded as downward deflections. Concentrations of GLU higher than 1.5×10^{-4} M often caused muscle contraction with microelectrode displacement.

The superfusion of the lobster fibre with glutamate (0.5-1.5 x 10^{-4} M) produced membrane depolarizations which reached a maximum within 2 min (Hironaka, 1974) Depolarizations induced by Kai (10^{-4} to 10^{-3} M) were smaller than those caused by glutamate, the action of which was potentiated by Kai in concentrations producing little or no depolarization alone (Figure 1).

In the frog cord, glutamate (5 x 10^{-5} to 10^{-3} M) and Kai (in lower concentrations of 10^{-5} to 10^{-4} M) evoked spike activity in the ventral roots with initial increase and subsequent depression of ventral root potentials. In the first 10 min glutamate (10^{-4} M) induced a 51% increase in the spinal ACh output while with Kai (10^{-4} M) a 112% rise was found. In still lower concentrations, neither Kai (5 x 10^{-6} M) nor glutamate (5 x 10^{-5} M) separately affected ACh output but when added simultaneously raised the ACh output by 30%.

These findings suggest that Kai has more potent effects than glutamate on the frog spinal cord but not on the lobster muscle fibre.

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Actions of γ-aminobutyric acid (GABA) on ganglionic transmission and ganglion cell excitability

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GABA increases Cl^- conductance in rat sympathetic ganglion cells and produces a membrane depolarization, probably because $E_{Cl} < E_m$ (Adams & Brown, 1973). We wish here to consider the consequences for ganglion cell excitability and transmission.

Isolated rat superior cervical ganglia were bathed in flowing oxygenated Krebs' solution at ambient temperature, and neurones impaled with microelectrodes filled with K citrate or acetate. Cells were stimulated directly by passing depolarizing current pulses through the microelectrode or orthodromically through a suction electrode into which the preganglionic trunk was drawn.

On the direct spike GABA (100 μM) (i) reduced the positive overshoot and rate of rise and (ii) increased the threshold depolarizing current. These effects could be explained quantitatively in terms of (a) the increased membrane conductance and (b) increased steady-state Na^+-inactivation during GABA-depolarization (Hodgkin & Huxley, 1952).

In neurones showing minimal impalement shunting (such that the recorded membrane potential exceeded −55 mV (Adams & Brown, 1975)) GABA did not usually block the transmission of single, supramaximal orthodromic stimuli: instead, the amplitude of the orthodromic spike was reduced to an extent comparable with that of the direct spike. This accords with previous observations with extracellular electrodes (De Groat, 1970; Bowery & Brown, unpublished observations). Although the amplitude of the synaptic potential is severely depressed by GABA, transmission does not fail because the synaptic potential is superimposed on a depolarization driving toward E_{Cl} such that the membrane potential attained by the synaptic potential still exceeds the threshold for spike generation.

In contrast, when the impalement leak short-circuited the membrane resistance sufficiently to reduce the resting potential below −55 mV, orthodromic transmission was invariably blocked by GABA. This might be an experimental artefact, reflecting increased Na^+-inactivation during the sustained impalement-depolarization with a consequent rise in the voltage threshold for spike generation. Thus, although attaining a lower absolute membrane potential than in undamaged cells, on adding GABA the synaptic potential is reduced below the now-elevated threshold for spike generation. In support of this hypothesis, prolonged depolarization (produced by either injected current or carbachol) elevated the voltage threshold and reduced the spike overshoot and rise rate as expected for increased resting Na^{+}-inactivation (Hodgkin & Huxley, 1952). Another consequence of pronounced Na-inactivation is the failure of these cells to give repetitive spikes during prolonged depolarization.

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L-2,4-diaminobutyric acid (L-DABA) as a selective marker for inhibitory nerve terminals in rat brain

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^{3}H-GABA is accumulated in glial cells of rat sensory ganglia by a high affinity uptake process, which like that in nerve terminal is temperature sensitive and requires the presence of sodium ions in the incubation media, (Schon & Kelly, 1974a and b). Furthermore, ^{3}H-GABA uptake into glial cells was shown to be potently inhibited by β-alanine, a poor inhibitor of ^{3}H-GABA uptake in nerve terminals. In contrast, another GABA analogue, L-DABA was a potent inhibitor of ^{3}H-GABA uptake into nerve terminals but was less effective on the uptake of ^{3}H-GABA into glia. Schon & Kelly (unpublished observations) later confirmed that β-alanine was a substrate for the GABA uptake process in glia of the rat sensory ganglia and cerebral cortex and suggested that this amino acid might prove useful as a specific maker for such glial sites. We have examined the alternative possibility that L-DABA is a specific substrate for the GABA uptake process in nerve terminals (Simon & Martin, 1973) but not for that in glial cells, and that L-DABA might thus prove useful in the identification of nerve terminals able to take up GABA.

This hypothesis was confirmed directly by using ^{3}H-DL-DABA. The accumulation of ^{3}H-DL-DABA into small prisms of rat cerebral cortex continued linearly for one h and appeared to be mediated by a saturable process with an apparent K_m of 31 μM and a V_{max} of 33 nmol g^{-1} min^{-1} at 25°C. Like the uptake of GABA, the uptake of DABA was sodium dependent, however an increase in the incubation temperature from 25°C to 37°C greatly enhanced the uptake of DABA but not that of GABA. DABA accumulation also exhibits the same chemical specificity as that for GABA and was potently inhibited by GABA (IC50 = 17 μM) and the GABA analogues, DL-3 hydroxy GABA (IC50 = 100 μM) and 3-fluoro-GABA and the mercurial, *p*-chloromercuriphenylsulphonic acid (IC50 = 19 μM) and unaffected by β-alanine (Test concentration = 1 mM).

Electron microscopic autoradiography showed that the ^{3}H-DABA accumulated by small prisms of cerebral cortex was localized predominantly over nerve terminals. Furthermore, when ^{3}H-DABA was injected from a fine glass microelectrode into a single lobule of the rat cerebellar vermis and the animal killed by perfusion with 5% glutaraldehyde in Krebs' solution, electron microscopic autoradiography showed labelling over small nerve terminals and neurone cell bodies in all layers of the cerebellum. More detailed analysis allowed many of the labelled constituents to be identified either as stellate cells, or axon terminals of Golgi or basket cells.

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Bioanalytical assay of clozapine and its N-oxide metabolite and the determination of their blood levels in the dog

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Clozapine (8-chloro-11-(4-methyl-1-piperazinyl)-5H-dibenzo-[b,e] [1,4] diazepine) is a neuroleptic drug, the special feature of which is its low propensity for causing extrapyramidal effects (Ayd, 1974). The N-oxide of clozapine has been shown to be the principal metabolite in the dog and in man (Gauch & Michaelis, 1971). Since N-oxidation of tertiary amines and subsequent reduction back to the corresponding base have been established as common metabolic routes (Bickel, 1969), this study was aimed at clarifying the oxidation and reduction processes which occur following administration of clozapine, by simultaneously measuring the pharmacokinetics of clozapine and its N-oxide metabolite in the blood of dogs.

None of the known bioanalytical methods based on gas-liquid chromatography, thin-layer plate chromatography, polarography or radioimmunoassay, was found to be sufficiently sensitive to detect and differentiate between clozapine and its N-oxide metabolite. In this study, a method using high-pressure liquid chromatography (HPLC) was developed for this purpose and then applied in some preliminary experiments in dogs.

The drug and its N-oxide metabolite were extracted from samples of blood and urine with chloroform. The condensed extract was applied with a sample loop to a column (250 x 3 mm) which was slurry-packed with the stationary phase Merckosorb SI 60 (10 μm). A gradient of chloroform/methanol/water served as the mobile phase. Using a Hewlett Packard instrument with u.v. detection, clozapine and its N-oxide could be determined to a minimum concentration of 10 ng/ml of blood or urine (5% significance level).

The blood levels of clozapine and clozapine-N-oxide were measured with this HPLC method in 2 beagles over periods of 48 h following administration. Each dog received on separate weeks 300 mg clozapine orally, 300 mg clozapine-N-oxide orally and either 50 mg clozapine or 50 mg clozapine-N-oxide i.v. Both clozapine and clozapine-N-oxide were rapidly absorbed after oral administration. Oxidation of clozapine and reduction of clozapine-N-oxide occurred in the blood very soon after either oral or intravenous administration. The reduction process was faster than the oxidation process; the maximum level of clozapine was observed 10 min after intravenous administration of the N-oxide, whereas the maximum level of the N-oxide was not measured until 1 h after intravenous administration of clozapine. The clozapine/clozapine-N-oxide ratio oscillated during the first 6 h after administration. No common continuous pharmacokinetic model could describe the observed data due to the inter- and intra-subject variations in the levels of the two compounds.

It is concluded that both clozapine and its N-oxide should be determined in future pharmacokinetic studies in the dog and in man since the two compounds are so readily converted to each other *in vivo*. The HPLC method described would be of value in this type of study.

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An NADPH dependent warfarin reductase in human and rat liver and kidney soluble fraction

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Warfarin alcohols are major plasma metabolites of patients maintained on normal racemic warfarin, being present at about one third of the plasma concentration of warfarin. They are formed by reduction of the ketone group in the warfarin molecule. This reduction creates a second asymmetric centre and therefore two diastereoisomeric alcohols may be formed, warfarin $alcohol_1$ and warfarin $alcohol_2$. Each diastereoisomer exists in two enantiomeric forms, namely DL and LD for $alcohol_1$ and DD and LL for $alcohol_2$. About five times more warfarin alcohol is found in the plasma of man after a 100 mg single dose of (D)-warfarin than after an identical dose of (L)-warfarin. After (D)-warfarin the alcohol found in the plasma is warfarin $alcohol_1$ which probably has the DL configuration (Chan, Lewis & Trager, 1972; Hewick & McEwen, 1973).

We have initiated investigations regarding the origin of the warfarin alcohols. Previous workers (Ikeda, Ullrich and Staudinger, 1968) reported the formation of a non-phenolic metabolite of racemic warfarin produced in the presence of rat hepatic soluble fraction. Using hepatic and renal soluble fraction from man and the rat we have detected a number of non-phenolic warfarin metabolites. These metabolites, based on similar fluorescence properties, u.v. absorption spectra and chromatographic mobility, appear to be warfarin alcohols.

Soluble fraction, prepared from fresh rat tissue (200 g female Wistar rats) or from human samples, obtained 3.5 to 10 h after death, was incubated with (D)- or (L)-warfarin and an NADPH generating system at 37°C for 50 minutes. Warfarin alcohols formed were assayed by the method of Lewis, Ilnicki & Carlstrom (1970) as modified by Hewick & McEwen (1973).

The reductase activities of the livers of both species were similar and were about one fifth of the activities found in the kidneys. (D)-warfarin was reduced 2-8 times more readily than (L)-warfarin giving mostly $alcohol_1$, whereas $alcohol_2$ was the main product of (L)-warfarin reduction. For the four tissues the apparent Km values for the reduction of (D)-warfarin were similar, ranging from 0.63 to 1.5 mM.

Warfarin reductase activity occurring in fresh human tissue may be about twice that found in our human *post mortem* samples since we found that in 7 h rat hepatic *post mortem* samples, the reductase activity had declined by almost 60%.

These findings indicate that the relatively high concentration of warfarin alcohol (probably in the DL configuration) found in human plasma after (D)- or racemic warfarin administration may be attributable, at least in part, to stereoselective reductase enzymes in the hepatic and kidney cytosol. Alcohol dehydrogenase is probably not involved since we find ten times more warfarin alcohol is formed using NADPH compared with NADH as cofactor. Warfarin reductase however does bear some resemblance to the ketone reductases reported by Leibman (1971) in that it also occurs in liver cytosol and is NADPH linked.

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The effect of a single dose of ethanol on the hepatic microsomal metabolism of foreign compounds in the rat

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The ability of ethanol administered chronically, to increase the metabolism of foreign compounds by the hepatic microsomal fraction is well documented. Relatively little is known however, of the effects of a single dose of ethanol upon the activity of the hepatic microsomal mixed function oxidase.

Ethanol 170 mmol/kg body weight, was administered as a 25% (v/v) solution in water to 250 g male Wistar rats by stomach tube. The hepatic microsomal fraction was prepared by the method of Ernster, Siekevitz & Palade (1962) in 0.25 M sucrose containing 0.05 M Tris buffer pH 7.4. The metabolism of foreign compounds was measured over 30 min at 37° C using the incubation conditions described by Mazel (1971). The formation of p-aminophenol from aniline was measured by the method of Schenkman, Remmer & Estabrook (1967) and formaldehyde formed from aminopyrine by the method of Nash (1953). Cytochrome b_5 and cytochrome P-450 were determined as described by Dallner (1963), NADPH-cytochrome c reductase by the method of Mazel (1971), NADPH-cytochrome P-450 reductase by the method of Gigon, Gram & Gillette (1969) and spectral changes produced by the interaction of foreign compounds with the microsomal fraction determined as described by Schenkman *et al.* (1967). Microsomal protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951).

A single dose of ethanol resulted in an increase in aniline hydroxylation, from 11.9 ± 0.6 nmol mg^{-1} 30 min^{-1} ($n = 8$) to a maximum of 19.8 ± 2.9 nmol mg^{-1} 30 min^{-1} ($n = 8$, $P < 0.05$) at 24 hour. Aminopyrine demethylation was simultaneously reduced from 124.2 ± 11.9 nmol mg^{-1} 30 min^{-1} ($n = 8$) to 48.4 ± 10.1 nmol mg^{-1} 30 min^{-1} ($n = 8$, $P < 0.01$). There was no change in the liver weight or in the yield of microsomal protein/g liver. The levels of microsomal cytochrome b_5, cytochrome P-450, NADPH-cytochrome c reductase and NADPH-cytochrome P-450 reductase were unchanged. There was however, a $29.0 \pm 6.5\%$ ($n = 4$, $P < 0.01$) decrease in the type I spectral change produced by 5 nM aminopyrine, whilst the type II spectral change produced by 5 mM aniline was reduced by only $10.0 \pm 3.2\%$ ($n = 4$, $P > 0.05$).

Cycloheximide 1 mg/kg, an inhibitor of drug induced protein synthesis (Miller & Jondorf, 1973) completely blocked the increase in aniline hydroxylation but could not prevent a decrease in aminopyrine demethylation. The increase in aniline hydroxylation was due to an increase in the Vmax whilst the Km was unchanged. The effects of ethanol on aniline hydroxylation were not additive with the effects of either of the classical inducers of the microsomal mixed function oxidase, phenobarbitone or 3-methylcholanthrene.

It is concluded that the decrease in the metabolism of aminopyrine produced by the administration of a single dose of ethanol may be associated with a decrease in the binding to the type I site on cytochrome P-450. It is not possible at present to propose a mechanism for the increase in the metabolism of aniline.

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The metabolism of ethyl, n-propyl, n-butyl and iso-amyl alcohols by the isolated perfused rat liver

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Ethanol, n-propanol, n-butanol and iso-amyl alcohol have been added to the fluid perfusing isolated rat livers, and their disappearance from the perfusate with time followed. Livers from young adult female rats (Wistar) weighing 180-240 g were perfused by the portal vein with 100 ml of freshly prepared 20% rat donor blood in Krebs solution equilibrated with 95% O_2, 5% CO_2 at 37°C. The flow rate of the perfusate was 20 ml/minute. The concentrations of the alcohols in the perfusate were assayed by gas-liquid-chromatography (Curry, Walker & Simpson, 1966; Bonnichsen & Linturi, 1962) using 4-methyl-1-pentanol as an internal standard.

As in man (Shumate, Crowther & Zarafshan, 1967) and in the intact animal (Makar & Mannering, 1970), the elimination of ethanol from the perfusate was biphasic. At low concentrations (below 5 mmol L^{-1}) it was exponential with time (half life 13.78 (± 2.28 s.e. mean) min), at higher concentrations it was linear (rate 10.5 (± 0.95 s.e. mean) $mmol.h^{-1}$). The three higher aliphatic alcohols were eliminated in a similar biphasic manner, though the rates of the linear and exponential phases differed from one alcohol to another, as did the concentration at which the exponential process changed to the linear process. Calculation of the apparent Michaelis constant (K_m) and maximal velocity (V_{max}) for ethanol in the system (Table 1) gave values for these parameters which were in excellent agreement with those already published for a similar isolated perfused liver system (Makar & Mannering, 1970). The apparent K_m and V_{max} values for the higher alcohols were compatible with those obtained with purified alcohol dehydrogenase (ADH) (Theorell & Bonnichsen, 1951; Winer, 1958).

TABLE 1 Apparent Michaelis constants (K_m) and maximal velocities (V_{max}) of ethanol in the isolated perfused rat liver system.

Alcohol added simultaneously with ethanol	*K_m of ethanol Moles*	*V_{max} of ethanol mmol/l/min*
None	2.23×10^{-3}	0.14
n-propanol	5×10^{-3}	0.14
n-butanol	4×10^{-3}	0.14
Iso amyl alcohol	8×10^{-3}	0.14

Simultaneous addition of ethanol (initial concentration 10 mM) and n-propanol (2-3 mM), n-butanol (1.9 mM) or iso-amyl alcohol (1.2 mM) to the perfusate inhibited both the linear and the exponential phases of ethanol elimination. The apparent K_m and V_{max} values for ethanol in this situation reveal that the inhibition is competitive (Table 1).

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The effects of intravenous injections of vinblastine or vincristine on the responses of the rat heart to nerve stimulation and to drugs.

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Intravenous injection of vinblastine (3 mg/kg) depletes noradrenaline (NA) levels in the rat heart, and reduces cardiac binding of ^{3}H-NA (Keen & Livinston, 1970, 1971; Cheney, Hanin, Massarelli, Trabucchi & Costa, 1973, Hanbauer, Kopin, Maengwyn-Davies, Thoa & Weise, 1973, Hanbauer, Jacobowitz & Kopin, 1974). There is evidence to suggest that this effect may be due to destruction of noradrenergic nerve terminals (Bennett, Cobb & Malmfors, 1973: Hanbauer *et al.*, 1974) It has been claimed that the action of vinblastine on noradrenergic neurons is not possessed by its chemical analogue, vincristine (Cheney *et al.*, 1973). Furthermore, neither vinblastine nor vincristine administered intravenously appear to affect cholinergic neurons (Cheney *et al.*, 1973). The effects of these drugs on the response of the rat heart to nerve stimulation, to NA and to acetylcholine (ACh), have now been investigated.

Male Wistar rats weighing between 250 and 300 g were used vinblastine sulphate or vincristine sulphate was dissolved in Kreb's solution (1 mg/ml) and injected into a tail vein to give a dose of 3 mg/kg. The rats were killed 30 h later; the left atrium was removed and set up in a 100 ml organ bath (containing Kreb's solution at 37° C bubbled with 95% oxygen and 5% carbon dioxide) and connected to an isometric recording system. The quiescent preparation was driven by transmural electrical pulses (4 Hz, 2 ms, 6 V); nerve stimulation was effected by increasing the strength of the stimulating pulses to 100 V (Blinks, 1966) for 15 seconds. Noradrenergic responses were observed in the presence of atropine (5×10^{-6} M), cholinergic responses were observed in the presence of propranolol (5×10^{-7} M).

Thirty hours after treatment with vinblastine (3 mg/kg) there was a 70% reduction in the positive inotropic effect of noradrenergic nerve stimulation ($n = 7$, $P < 0.025$), and, although there was a marked reduction in the force of contraction of the left atrium, its sensitivity to NA was increased 12-fold ($n = 8$; $P < 0.001$) Vincristine (3 mg/kg) caused a 65% reduction in the effect of noradrenergic nerve stimulation ($n = 7$; $P < 0.05$, and a 42-fold increase in sensitivity to NA ($n = 8$, $P < 0.001$), as with vinblastine, the absolute force of contraction of the atrium was markedly reduced. Neither drug caused a significant change in the effects of cholinergic nerve stimulation nor in the response to ACh ($n = 10$).

The present findings corroborate the report of differential effects of vinblastine and vincristine on noradrenergic and cholinergic neurons (Cheney *et al.*, 1973), but indicate that both drugs can affect noradrenergic neurons.

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Mechanisms of concanavalin A-induced inflammation in the rat

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Concanavalin A (Con A), a mitogenic lectin derived from the jack bean, has been shown to stimulate lymphocytes to produce soluble mediators known as lymphokines (Pick, Brostoff, Krejci & Turk, 1970). Lymphokines have been implicated in the mediation of chronic inflammatory processes (e.g. Pick & Turk, 1972) and consequently it was of interest to establish (a) whether Con A induced an inflammatory response after administration to the rat hind paw and (b) whether the substances mediating the response resembled those produced by more widely used phlogogenic agents.

Male Wistar rats (CE/CFHB strain, 80-100 g) were used in these experiments. Con A was dissolved in 0.9% saline (w/v) and injected in 0.1 ml volumes via the subplantar route into the right hind paw. The contralateral paw received an equal volume of saline. The mean difference between both paws, as measured by mercury displacement plethysomography, was determined at various time intervals.

Con A produced a dose related (0.01-1.0 mg/paw) paw oedema that was slow in onset reaching a maximum by 24 h and still present at 72 hours. Both cyproheptadine (10 mg/kg, s.c.), a histamine and 5-hydroxytryptamine (5-HT) antagonist, and methysergide bimaleate (2.5 mg/kg, s.c.), a 5-HT antagonist, administered separately 30 min before Con A significantly suppressed Con A–induced oedema up until 3 hours. However, the antihistamine, mepyramine maleate (2.5 mg/kg, s.c.) administered 30 min before Con A, was without effect. Soyabean trypsin inhibitor (SBTI; 80 mg/kg, s.c.) which depresses kinin levels (Webster, Maling, Zweig, Williams & Anderson, 1972), administered 30 min prior to Con A inhibited the oedema almost completely up to 6 hours. Moreover, in separate experiments, kinin-like activity was measurable from air-blebs produced by the injection of air (after Willis, 1969) followed by 7.5 mg Con A in 5 ml saline. This was maximal at 16 hours.

Indomethacin (5 mg/kg) and aspirin (200 mg/kg), anti-inflammatory drugs capable of inhibiting prostaglandin production, administered subcutaneously 60 min before Con A both inhibited the oedema. This inhibition was approximately 30% for both drugs at 4 hours. Prostaglandin-like activity is also measurable by bioassay on the rat fundic strip (Piper & Vane, 1969) in extracts from Con A-air blebs (7.5 mg per rat) taken at intervals over a 24 h period and was maximal at 16 hours.

Thus 5-HT, kinins and prostaglandins appear to act as mediators of Con A-induced oedema although histamine does not appear to play a role in this response. Since a combination of cyproheptadine, SBTI and indomethacin does not completely abolish the Con A oedema this residual response is likely to be mediated by other factors such as complement and constituents of cellular infiltrates that are not affected by such drug treatment.

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AH 10407: A novel, short-acting, competitive neuromuscular blocking drug in animals and man

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It has been possible to obtain neuromuscular blocking drugs which are short-acting in animals, e.g. γ-oxalolaudonium (Brittain, Collier & D'Arcy, 1961), AH 8165 (Brittain & Tyers, 1973) and stercuronium (Hespe & Wieriks, 1971), but these drugs have a much more prolonged action in man (Blogg, Savege, Simpson, Ross & Simpson, 1973; Admiraal, 1973). A new compound, 1,1^1-azobis [3-methyl-2-phenylbenzimidazolinium] dichloride, AH 10407 (Glover, Rowbottom & Bishop, 1973), has now been shown to be a short-acting, competitive, neuromuscular blocking drug in both animals and human beings. We wish to report the pharmacology of this compound and to discuss the basis for its brevity of action.

In the anaesthetized cat AH 10407, 0.4-1.6 mg kg^{-1} i.v., caused a dose-dependent neuromuscular block of the indirectly stimulated (1 Hz) tibialis muscle (28 ± 2.7 to 94 ± 4.2%) which lasted from 14-45 seconds. The onset of block following single doses was very rapid (5-10 sec). In addition, when AH 10407 was infused intravenously at 0.5 mg kg^{-1} min^{-1} for 1 h, recovery from the block (97%) was complete within 60 sec of stopping the infusion. The neuromuscular blocking action of AH 10407 was rapidly and completely reversed by neostigmine, 0.05 mg kg^{-1} i.v.

In the anaesthetized cotton-eared marmoset, a primate which closely resembles man regarding the duration of action of neuromuscular blocking drugs, AH 10407, 2 mg kg^{-1} i.v., caused a 95% block of tibialis muscle twitches (f = 1 Hz) which lasted for 3 minutes. After toxicity studies in several animal species AH 10407 was investigated for its neuromuscular blocking action in conscious human volunteers. Using the ulnar nerve-adductor pollicis muscle preparation of the 'isolated' forearm, AH 10407, 0.4 mg i.v., caused a 63% block of muscle twitches which recovered in 1.1 min after removal of the tourniquet.

AH 10407 is rapidly degraded in the presence of bicarbonate ions. For example, in Krebs solution 83 ± 2.5% of AH 10407 is degraded within 1.5 min at 37°C and in human blood 90 ± 3.1% is degraded within 1.5 minutes. In neutral and acidic solutions AH 10407 is relatively stable. Therefore, it is probable that the very short duration of action of AH 10407 is due to base-catalyzed chemical degradation brought about by a nucleophilic attack by bicarbonate ions and other basic ions on the benzimidazolinium nucleus.

Although the inherent instability of AH 10407 results in problems in its chemical development and pharmaceutical formulation, the concept of designing molecules that are susceptible to base-catalyzed degradation would make possible a neuromuscular blocking drug that is reliably short-acting in man.

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The uptake and subcellular distribution of ^{3}H-choline by the retina

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It has been known for a long time that the vertebrate retina but not the optic nerve, contains acetylcholine (ACh) and possesses exceptionally high choline acetyltransferase (ChAc) activity (Feldberg & Mann, 1946; Hebb, 1963). Furthermore, it has been claimed that the retina has a higher binding capacity for dimethyl-d-tubocurarine than other areas of the cat central nervous system (De Robertis & Fiszer, 1968). Although these results strongly indicate a cholinergic mechanism in the retina, the location and function of the presumed cholinergic synapses is unknown.

In order to obtain further information on the retinal cholinergic system, we have examined the uptake and subcellular distribution of ^{3}H-choline by the retina.

Isolated rat or rabbit retinae were given a preliminary incubation in Krebs bicarbonate Ringer at 37°C for 10 minutes. ^{3}H-Choline was then added to give a final concentration of 6.5×10^{-8} M and the incubations were continued for various times. The retinae were then recovered and washed. The accumulated radioactivity was estimated by liquid scintillation counting after dissolving the tissue in Soluene.

Rat and rabbit retinae rapidly accumulated radioactivity and after 45 min incubation, tissue/medium ratios of approximately six were achieved. Preliminary metabolic studies indicated that about 50% of the labelled choline was converted to ^{3}H-acetylcholine during 30 min incubation. Kinetic analysis of the uptake process suggested that it could be resolved into two components: a low affinity process (apparent $K_m = 100\ \mu$M) and a high affinity process ($K_m = 2.15\ \mu$M). The high affinity uptake was temperature-dependent (T/M = 0.7 at 0°C) and was reduced by about 50% in the absence of sodium or by the presence of hemicholinium (0.1 mM).

The subcellular distribution of the radioactivity accumulated by rabbit retinae incubated with a low concentration of ^{3}H-choline (6.5×10^{-8} M) was studied on continuous sucrose density gradients (Neal & Atterwill, 1974). More than 80% of the radioactivity appeared to be localized in particles which had a median equilibrium density equivalent to 1.24 M sucrose. This 'bound' radioactivity was reduced by 50% following exposure of the particles to hypo-osmotic conditions. Over 85% of the ChAc activity measured on the same gradients was also present in particles which had the same equilibrium density as those accumulating labelled choline.

It is concluded that the retina possesses a high affinity uptake process for choline. This process is present in particles which may prove to be retinal cholinergic nerve endings.

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Drug desensitization and cross-desensitization in guinea-pig ileum

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Drug desensitization was assessed in guinea-pig ileum strips suspended in Ringer solution employing isometric recording. An electronic compensating device (Schild & Seaford, 1970) automatically readjusted the length of the muscle thus ensuring a constant initial tension. Desensitizing drugs were administered for a period of 8-10 min in a high concentration (50-100 times that needed to establish a dose-response curve) and the degree of subsequent desensitization was expressed in terms of dose-ratio usually 10 min afterwards.

The main findings were as follows: 1. Degree of desensitization depended on concentration and period of contact with the desensitizing drug. 2. Strong cross-desensitization was seen after acetylcholine desensitization of histamine responses (ACh → Hi); less strongly by Hi → ACh: strong cross-desensitization by Hi → 5-HT; little or none by 5-HT → Hi: strong cross-desensitization by Hi → bradykinin (BK). 3. The degree of desensitization was related to the Ca^{++} content of the Ringer solution *during* the desensitization period (using hepes Ringer, Good, Winget, Winter, Connolly, Izawa & Singh, 1966, which does not precipitate calcium, as the desensitizing solution). When the desensitizing solution contained 4 mM Ca^{++} in place of 0.2 mM Ca^{++}, duration and degree of both auto-desensitization Hi → Hi (Schild, 1973), ACh → ACh, BK → BK and cross-desensitization ACh → Hi, Hi → BK were significantly diminished. 4. Desensitization Hi → Hi was obtainable in the complete absence of calcium in solution suggesting that calcium is not required for the interaction of histamine with receptors.

These findings suggest that drug desensitization in guinea pig ileum may, depending on the drug used, be unspecific or drug-specific and that it is related to the calcium content of the desensitizing solution. It seems possible that the effect of calcium is exerted at a post-receptor level common to different drugs.

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The effect of isoprenaline on ^{86}Rb uptake by horse lymphocytes *in vitro*

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Catecholamines affect a number of functions of leucocytes; these functions include inhibition of histamine release (Lichtenstein & Margolis, 1968), inhibition of immune cytolysis (Henney, Bourne & Lichtenstein, 1972) and inhibition of sheep red blood cell plaque formation (Melmon, Bourne, Weinstein, Shearer, Brauminger & Kraur, 1974).

The possible importance of changes in cation distribution during mitogen-induced transformation of lymphocytes (Quastel & Kaplan, 1970; Allwood, Asherson, Davey & Goodford, 1971) led us to carry out a study on the effect of isoprenaline on the uptake of ^{86}Rb by lymphocytes.

Lymphocytes were isolated from horse peripheral blood. To remove granulocytes, the lymphocyte-rich plasma, obtained by gravity sedimentation for 15 min, was passaged through three (7.0 x 1.4 cm) glass wool columns set up in series. The lymphocyte-rich effluent obtained from these columns was run onto a fourth set of (12.0 x 1.4 cm) glass wool columns which were incubated at 37° C for 1 hour. Elution of these latter columns with medium 199 (pH 7.4, gassed with 5% CO_2 in air) followed by lysis of the remaining red cells with 155 mM NH_4Cl gave a fraction of lymphocytes (always greater than 97% pure from other cell types), which were suspended in medium 199 containing 20% heat-inactivated homologous serum at a concentration between $2\text{-}10 \times 10^6$ cells ml^{-1}.

The lymphocytes obtained by this method were maintained on a culture-tube roller, without gassing, at 37° C and were allowed to recover for at least 1 hour. After this time they appeared morphologically normal under light and electron microscopy, excluded dyes such as trypan blue and eosin Y, showed an increased uptake of ^{14}C-thymidine in the presence of 20 μg ml^{-1} phytohaemagglutinin (PHA) and exhibited 50% inhibition of ^{86}Rb uptake with 10^{-5} M ouabain.

Cells were incubated at 37° C for 15 min in the presence of ^{86}Rb and were removed from the surrounding medium by spinning for 1 min in a microcentrifuge at 12,500 g. Correction for any remaining medium was calculated using ^{14}C-sorbitol. When isoprenaline was administered at a range of concentrations (10^{-4}-10^{-10} M) a significant ($P < 0.01$) increase in ^{86}Rb uptake over control was seen with a maximum at 10^{-7} M.

Isoprenaline has been shown to increase significantly the synthesis of IgG by human peripheral lymphocytes over the range 10^{-7}-10^{-10} M, with a maximum at 10^{-8} M (Sherman, Smith & Middleton, 1973). Therefore it would seem that a peak activity of isoprenaline at 10^{-7} M for ^{86}Rb uptake may not be unrelated to another isoprenaline effect on lymphocytes. However, the importance of the role of cations in catecholamine–stimulated cellular activity still has to be elucidated.

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Stimulant effects of 5-hydroxytryptamine on cardiac sympathetic nerves

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5-Hydroxytryptamine (5-HT) stimulates the isolated rabbit heart and its effects are abolished by pretreatment with reserpine (Jacob & Poite-Bevierre, 1960) suggesting mediation by catecholamine release. This action has now been further investigated. In particular, 5-HT has been compared with noradrenaline, which acts directly on the receptors, with tyramine, which releases noradrenaline by stoichiometric displacement from the neurone (Trendelenburg, 1972) and with dimethylphenylpiperazinium (DMPP) which releases noradrenaline by depolarization resulting from activation of nicotinic receptors (Muscholl, 1970).

Hearts were removed from rabbits given heparin (500 μ/kg) 5 min before killing and perfused at constant pressure by the Langendorff technique with Tyrode solution containing atropine (5×10^{-7} g/ml) at 37°C. Right atrial and ventricular tensions and right ventricular rate were recorded as previously described (Fozard & Muscholl, 1971). Drugs were either given by bolus injection or incorporated into the perfusion fluid.

Noradrenaline (0.04-40 μg), 5-HT (0.5-512 μg), DMPP (5-320 μg) and tyramine (2.5-640 μg) caused dose-dependent increases in the rate and force of cardiac contraction. Propranolol reduced these responses at low concentrations. Using the rate response, the pA_2 values obtained for the antagonism of noradrenaline, 5-HT, DMPP and tyramine by propranolol were 8.42 ± 0.08, $n = 8$; 8.43 ± 0.24, $n = 5$; 8.45 ± 0.16, $n = 3$; 8.29 ± 0.12, $n = 3$ respectively. Pretreatment of animals with 6-hydroxydopamine (Fozard, Kelly & Small, 1973) markedly reduced responses of the hearts to 5-HT, DMPP and tyramine despite an increase in the sensitivity to noradrenaline.

Tachyphylaxis developed rapidly to 5-HT and

DMPP. During full tachyphylaxis to 5-HT normal responses to noradrenaline, DMPP and tyramine could be obtained. In contrast, with the hearts desensitized to DMPP, responses to 5-HT were also abolished, although those to noradrenaline and tyramine were little affected. Colchicine (10^{-4} to 10^{-3} g/ml) inhibited responses to 5-HT and DMPP concentration-dependently, but had no significant effects on responses to noradrenaline or tyramine.

After the cardiac noradrenaline stores were labelled by perfusion with ^{3}H-(−) noradrenaline (10 ng/ml—Starke, 1971), bolus injections of 5-HT (512 μg), tyramine (40 μg) and DMPP (40 μg) evoked tritium release from the hearts. The pattern of tritium appearance in the perfusate after 5-HT showed a peak, 10-20 s after the injection with little release being evident after 1 minute. A qualitatively identical pattern was obtained with DMPP. In contrast, tyramine released tritium at a constant rate during the 3 min period immediately following the injection. Reducing the Tyrode calcium ion concentration from 3.6-0.2 mEq/l did not affect the tritium release after tyramine, although the release evoked by 5-HT and DMPP was markedly inhibited.

The results confirm the suggestion that 5-HT stimulant responses on the rabbit heart are the result of noradrenaline release. They further suggest that the site of release is the terminal sympathetic nerve network. The mechanism of release shows more similarities to the DMPP release mechanism (depolarization) than to that of tyramine (neuronal uptake and stoichiometric displacement).

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5-Hydroxytryptamine synthesis in the isolated perfused rat brain

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Isolated perfused organs provide an experimental system for the study of metabolism in an organ retaining its structural integrity. Little work has been done upon monoamine metabolism in the perfused brain. We have developed a method for the *in situ* perfusion of rat brain which excludes the influence of extracranial tissues on tryptophan metabolism and this paper describes the use of this method in the study of 5-hydroxytryptamine metabolism.

Rat brains were perfused using the method described by Woods, Graham & Grahame-Smith (1974). The metabolic, and histological properties of this preparation are very similar to those observed *in vivo*. For example, the rate of glucose uptake was 0.79 μmol min^{-1} gram^{-1} in the presence of 5 mmol/l glucose, and the rate of acetoacetate uptake was concentration dependent being 0.14 μmol min^{-1} gram^{-1} after loading with 1 mmol/l acetoacetate and 0.24 μmol/min^{-1} gram^{-1} with 2 mmol/l.

For the study of 5-HT synthesis rats were anaesthetized with Nembutal (60 mg/kg i.p.) and the brains perfused with a medium containing glucose (10 mmol/l) together with tranylcypromine (1 mmol/l) and tryptophan. (0.1 or 1.0 mmol/l). After perfusion for varying times the brains were rapidly removed from the skull and stored at −20° C before determination of 5-HT and tryptophan concentrations.

Anaesthesia and preparation of the brain for perfusion resulted in a small increase in brain 5-HT concentration when compared with brains obtained after cervical dislocation (from 0.46 to 0.53 μg/g).

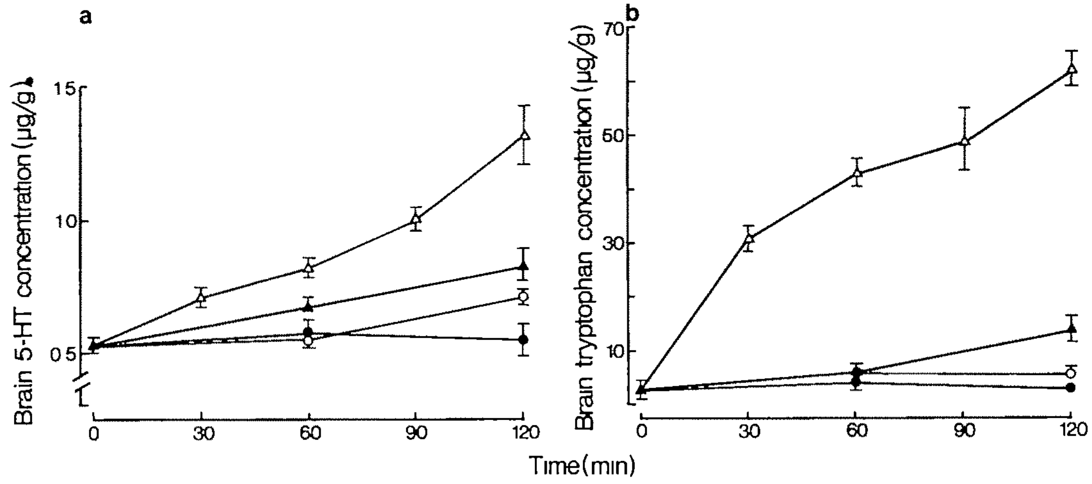

Figure 1 Brain 5-Hydroxytryptamine (Figure 1a) and L-tryptophan (Figure 1b) concentrations in the isolated perfused rat brain. Brains were perfused with either the basal medium which contained glucose (10 mmol/l) alone (●—●) or a medium containing tranylcypromine (1 mmol/l) (○—○). In other experiments the medium contained tranylcypromine (1 mmol/l) + L-tryptophan (0.1 mmol/l) (▲—▲) or tranylcypromine (1 mmol/l) + L-tryptophan (1.0 mmol/l) (△—△). Each point represents the mean ± S.E.M. for 3-7 observations.

Brain 5-HT concentrations were maintained at the control values (0.53 μg/g) during perfusion for 2 h with the basal medium which contained no added tranylcypromine or tryptophan (Figure 1a) (the initial tryptophan concentration being < 2 μg/ml). Addition of tranylcypromine (1 mmol/l) caused increase in brain 5-HT during the second h at a rate of 0.16 $\mu g\ g^{-1}\ h^{-1}$. When tryptophan was added with tranylcypromine the rate of 5-HT accumulation increased with tryptophan concentration being 0.16 $\mu g\ g^{-1}\ h^{-1}$ with 0.1 mmol/l and 0.4 $\mu g\ g^{-1}\ h^{-1}$ with 1.0 mmol/l.

Following perfusion with tryptophan the brain tryptophan concentration rose, the rate of accumulation increasing with the increasing initial concentrations (Figure 1b).

These results compare favourably with rates of 5-HT accumulation measured *in vivo* following loading with 100 mg/kg tryptophan. This model provides a means of studying brain 5-HT metabolism in a situation where there is a high degree of control over the experimental conditions.

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Distribution of chlorpromazine and its metabolites in subfractions of rat brain

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The distribution of chlorpromazine (CPZ) in the central nervous system was measured autoradiographically in the cat by Cassano, Sjöstrand & Hansson (1965) using large amounts of ^{35}S-CPZ. Earlier work by Sjöstrand, Cassano & Hansson (1965) investigating whole body distribution of CPZ in mice showed a concentration of label in the cerebral and cerebellar cortex. In order to study the area and subcellular distribution, rats were injected (8 mg/kg i.p.) with ^{3}H-CPZ at various times before being killed. Brains were removed, divided into cortex, mid-brain and hind-brain, all subsequent procedures being carried out on each area independently. Subcellular fractions were prepared from a 10,000 *g* pellet on a discontinuous sucrose gradient based on a method by Marchbanks & Whittaker (1967) and purity of fractions ascertained by electron microscopy. Radioactivity of each sample was measured by liquid scintillation counting Subsequent experiments measuring the ability of isolated subfractions to accumulate CPZ were attempted by incubating the subfractions in medium containing 10^{-4} M ^{3}H-CPZ at 37°C for 15 minutes. The reaction was terminated by centrifugation, the supernatant decanted, tubes wiped dry and pellet resuspended in 10% Triton X100. Samples were counted by liquid scintillation counting as in the earlier experiments.

A time course study following the injection of CPZ indicated an initial rise in all areas of rat brain rising to a maximum at about 15 min and then levelling off from 20-30 min, the highest concentration accumulating in the cortex and the smallest in the hind brain. There were surprisingly high levels in the membrane/myelin fraction compared to that in the synaptosomes in all areas, which was apparent after 15 minutes. It was found that there was a differential distribution of CPZ in the subfractions in the different areas at 10 min and 60 min after injection. At 10 min a relatively high concentration was found in the mitochondria which may be explained by our earlier work (Livingston & Phillips, 1974) on the effects of CPZ on oxidative phosphorylation in synaptosomes and mitochondria, although after 60 min the highest concentrations were found in the membrane/myelin fraction.

Experiments in which CPZ was incubated with subcellular fractions for 32 min showed similar results to whole animal experiments incubated for 60 min, the main difference being in the hind brain where there was a high accumulation in the synaptosomes relative to the other subfractions. However, in the cortex and mid-brain the previous finding of a high concentration in the membrane/myelin subfraction is reinforced, there being approximately twice as much CPZ accumulated in this fraction as in the synaptosomes and mitochondria in the cortex.

The relatively high concentration of CPZ found in the membrane/myelin fraction poses an interesting question as to the site of action of this drug in the central nervous system.

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Change in sensitivity to pentobarbitone and halothane induced by acute administration of central nervous system depressant drugs

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A withdrawal hyperexcitability, as manifested by a reduction in the duration of anaesthesia produced by an intracerebroventricular (i.c.v.) injection of pentobarbitone is seen in rats abruptly withdrawn from a number of central nervous system (CNS) depressant drugs following their chronic administration (Stevenson & Turnbull, 1974). However, since such experiments are necessarily time-consuming and because each animal can only be used once, we have investigated the possibility that CNS excitability might be altered by administration of large doses of depressant drugs over a much shorter period of time and have assessed the usefulness of other indices of CNS excitability.

First we attempted to keep male rats anaesthetized for approximately 8 h by beginning an i.p. infusion of pentobarbitone (30 mg kg^{-1} h^{-1}) immediately animals had lost their righting reflex following an i.p. injection of 50 mg/kg pentobarbitone. The rats were killed on awakening and the brain barbiturate levels were determined. Higher brain levels (23.6 ± 1.7 (5) mean ± s.d. μg/g) were found in these animals compared with rats killed on awakening after a single i.p. injection of 50 mg/kg administered at such a time that the animals awakened at approximately the same time as those given pentobarbitone by infusion (16.7 ± 3 2 (4); $P < 0.01$). Because of the high mortality (>60%) occurring during the infusion and the possible presence of barbiturate metabolites in the brain extract this experiment was not extended to include other drugs.

Secondly, female rats were kept anaesthetized for approximately 12 h by repeated i.p. injections of pentobarbitone. A typical dose regime was 25 mg/kg followed 2, 5 and 10 h later by further injections of 20 mg/kg. The next day, approximately 12 h after animals had regained their righting reflex, the duration of anaesthesia and brain barbiturate level on awakening was determined following an i.c v. injection of 800 µg [^{14}C]-pentobarbitone. Compared with saline pre-treated control animals, pentobarbitone-treated animals slept for a shorter period of time (control 6.9 ± 1 8 (7); treated 4.0 ± 1.7 (6) min. $P < 0.01$) and awakened with higher brain barbiturate levels (control 21.7 ± 7.7 (7); treated 35.2 ± 13.2 (6) µg/g. $P < 0.05$).

However, without using large numbers of animals, it is not possible to determine the time course of such change in CNS sensitivity. We therefore decided to measure the duration of halothane-induced anaesthesia in drug pre-treated animals in the hope that the change in CNS excitability might be more easily followed. Repeated injection of pentobarbitone for 10 h, as outlined above, resulted in the development of tolerance to halothane which was maximal 24 h after the last pentobarbitone injection. Tolerance was followed by a 'rebound' hypersensitivity to halothane which reached a maximum 42 h after pentobarbitone. Sensitivity had returned to normal by the third day. Similar patterns of change in sensitivity to halothane, i.e a post-tolerance hypersensitivity, were also observed after repeated injections of amylobarbitone and meprobamate. It is hoped to extend this work to include other centrally active drugs, particularly those on which physical dependence may develop.

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Effects of dexamethasone on phenylethanolamine N-methyltransferase (PNMT) and adrenaline (A) in the brains of adult and neonatal rats

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Recently the regional distribution of PNMT in rat brain has been demonstrated by immunohistofluorescence (Hökfelt, Fuxe, Goldstein & Johansson, 1974) and biochemical techniques (Saavedra, Palkovits, Brownstein & Axelrod, 1974). Only small amounts of A have been detected in rat brain stem (Gunne, 1962). The purpose of this study was to determine (1) the distribution of PNMT in rat brain; (2) the amounts of A in those regions rich in PNMT; (3) the sensitivity of PNMT and A to glucocorticoid hormone treatment.

Sprague-Dawley rats, both newborn and adult, were used. PNMT was assayed by a modification of the method of Axelrod (1962). For catecholamines, an enzymatic method capable of detecting picogram quantities of amine was employed (Cuello, Hiley & Iversen, 1973). The sensitivity for A was 50-75 pg, amounts that gave values of twice the blank. Dexamethasone was injected s.c. (1 mg/kg for adults and 0 1 mg/kg for newborn rats).

Highest PNMT activity was recorded in the medulla in a region bounded by a transverse section at the obex and a transverse section 3 mm rostral to the obex (0.454 ± 0.052 p mol product.mg wet wt.$^{-1}$ h^{-1}, mean ± s.e.m. n = 4). The hypothalamus contained 0.194 ± 0.012 p mol product.mg wet wt.$^{-1}$ h^{-1}. Small amounts of activity were recorded in midbrain, pons and spinal cord, but none in olfactory bulb, olfactory tubercle, septum, striatum, cerebellum, cerebral cortex or hippocampus. Only traces of A could be detected in the medulla, whereas the hypothalamus contained 0.04 ± 0.01 µg/g wet wt. (mean ± s.e.m. n = 4).

Dexamethasone treatment of adult male rats daily for 13 days increased PNMT both in the medulla (from 0.440 ± 0.092 to 0.792 ± 0.100, $P < 0.05$, $n = 6$) and in the hypothalamus (from 0.168 ± 0.056 to 0.372 ± 0.072, $P < 0.05$, $n = 6$). In the hypothalamus, A increased from 0.05 ± 0.01 to 0.08 ± 0.01 µg/g wet wt. ($P < 0.05$, $n = 6$). No change was seen in noradrenaline or dopamine concentrations of medulla or hypothalamus. A single dexamethasone injection did not change PNMT after 24 hours. Five daily injections increased PNMT by 56%, but only in the medulla. Five daily injections of dexamethasone to newborn rats had no effect on PNMT in hypothalamus or medulla, but caused a 14-fold increase in PNMT and a 30-fold increase in A in the superior cervical ganglion.

These observations are consistent with the hypothesis that the cell bodies of PNMT and adrenaline containing neurones are present in the medulla and that they send axons to terminate in the hypothalamus. The PNMT contained in the cell bodies appears to be inducible by glucocorticoid hormone, and the induced enzyme is transported to the hypothalamus, resulting in an increased A content. •

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Release of ^{3}H-(−)-noradrenaline from guinea-pig hypothalamic slices: effects of adrenoceptor agonists and antagonists

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The release of noradrenaline from peripheral adrenergic nerves in response to electrical stimulation is enhanced by α-adrenoceptor antagonists and reduced by α-adrenoceptor agonists. These effects have been attributed to the presence of α-adrenoceptors on the terminal axons through which transmitter release is inhibited. The present experiments were designed to investigate whether the release of noradrenaline from hypothalamic slices of the guinea-pig was similarly affected by drugs acting on α-adrenoceptors.

Slices of hypothalamus were incubated with ^{3}H-(−)-noradrenaline, then repeatedly washed with Krebs-Henseleit solution until the rate of efflux of tritium from the tissue had fallen to a low level (approx. 1 h): then, the residual tritium was present as ^{3}H-noradrenaline. Stimulation of the slice with biphasic pulses at 10 Hz for 30 s resulted in an increase in tritium efflux. With successive periods of stimulation at 30 min intervals, there was a steady decrease in stimulation-induced efflux.

The effects of drugs on resting and stimulation-induced effluxes of tritium were determined by adding them 19 min before the second period of stimulation. When α-adrenoceptor agonists were used, cocaine (100 µM) was present to prevent their uptake and the displacement of labelled noradrenaline.

Noradrenaline decreased stimulation-induced efflux in a concentration-dependent manner in the range 2-50 µM. Dopamine was approximately equipotent with noradrenaline; adrenaline was less potent, the threshold concentration being above 5 µM; isoprenaline had no significant effect in concentrations up to 50 µM.

Piperoxane (10 µM) increased stimulation-induced efflux, but phenoxybenzamine (10 µM) and phentolamine (10 µM) had no effect. However, phentolamine (5 µM) antagonized the effect of noradrenaline (20 µM) in reducing stimulation-induced efflux.

The findings suggest the presence in the hypothalamus of α-adrenoceptors through which stimulation-induced release of noradrenaline from stores in the tissue can be inhibited, but feed-back modulation of release by transmitter noradrenaline impinging on these receptors appears to be less important than in peripheral adrenergic neurones.

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Prevention of DOCA saline hypertension by central 6-hydroxydopamine; role of saline intake

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Destruction of central noradrenergic neurones by intracisternal (i.c.) injection of 6-hydroxydopamine (6-OHD) prevents the onset of renal hypertension in rabbits, (Chalmers, Dollery, Lewis & Reid, 1974) DOCA saline and spontaneous hypertension in rats (Haeusler, Finch & Thoenen, 1972). Central noradrenergic neurones may initiate the rise in arterial pressure in experimental hypertension. However, since food and water appetite is diminished in animals treated with central 6-OHD, an alternative explanation might be that these animals are salt depleted and show less rise in arterial pressure for this reason. Breese, Cooper & Smith (1973) noted DOCA saline rats treated with i.c. 6-OHD consume less saline than intact DOCA saline rats.

We have investigated the role of saline consumption in the effect of i.c. 6-OHD on this type of hypertension by restricting saline intake in a group of rats implanted with DOCA (30 mg pellet). Rats in this group D ($n = 10$) were given saline equivalent to the average consumption of rats in group C ($n = 10$) which had been pretreated with intraperitoneal pargyline (40 mg kg) and i.c. 6-OHD (200 μg x 2) and implanted with DOCA. Two control groups were used, a normotensive group A ($n = 8$) without DOCA implant and an hypertensive group B ($n = 7$) with DOCA implant. All rats were uninephrectomized and given only 0.9% saline to drink

At 5 weeks after implantation of DOCA the systolic arterial pressures in the groups were: A 140.4 ± 3.5, B 200.4 ± 6.9, C 150.1 ± 3.3, D 190.9 ± 5.4 (mmHg ± SEM). Saline consumption in Group C was significantly diminished throughout, and during the 5th week the consumption in Group C was 29.3 ± 5.0 ml/100 g/24 hrs compared with 44.1 ± 6.1 ml/1000 g/24 hrs in Group B. However, restriction of Group D to this intake did not prevent the hypertension.

Hence although saline intake is diminished in rats centrally depleted of noradrenaline, this is not the explanation for the failure to develop hypertension. Central noradrenergic neurones play a more direct role in maintaining arterial pressure.

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Loss of noradrenergic and dopaminergic terminals in the chronically isolated cerebral cortex of the cat.

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The increased epileptogenicity of chronically isolated cortex is most probably a consequence of denervation. One possibility is that denervation suppresses inputs of extracortical origin that normally exert a tonic inhibitory control on cortical excitability. Amongst the transmitter candidates for such an inhibitory control other than GABA, the authors have studied catecholamines (CA) in the cat cerebral cortex.

The synthesis of ^{3}H-noradrenaline (NA) and ^{3}H-dopamine (DA) from ^{3}H-tyrosine estimated in slices of the suprasylvian gyrus and of the cerebellar cortex revealed that the ^{3}H-DA/^{3}H-NA ratio was higher in the cerebral cortex than in the cerebellum. The uptake of ^{3}H-DA estimated in homogenates of the suprasylvian cortex was not only partially blocked by desipramine but also by benztropine (blocker of CA uptake in dopaminergic terminals). These data suggest that catecholaminergic innervation in the cerebral cortex of the cat can be attributed to dopaminergic as well as noradrenergic terminals and are in agreement with recent biochemical and histochemical results which clearly indicated the existence of DA terminals in the rat cerebral cortex.

In further experiments 1-^{3}H-dopamine uptake was estimated in homogenates of the chronically isolated suprasylvian gyrus and compared to that of the contralateral side. ^{3}H-dopamine uptake was markedly reduced 27 days after the operation and the residual ^{3}H-amine uptake was not sensitive to benztropine or desipramine. 2-^{3}H-CA synthesis from ^{3}H-tyrosine was no more detectable in slices of the chronically isolated suprasylvian gyrus (27 days). Thus these two techniques lead to similar results both types of catecholaminergic terminals degenerate in the chronically isolated area and dopaminergic as well as noradrenergic terminals, are exclusively of extracortical origin. This latter fact suggests the absence of dopaminergic interneurons in the cat neocortex.

The authors discuss the possibility that this dramatic decrease in the CA available, secondary to deafferentation, could be in part responsible for the augmented duration of the epileptiform after discharge in the chronically isolated cortex.

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The effect of dopamine receptor stimulants on locomotor activity and cyclic AMP levels in the rat striatum

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Stimulation of central dopamine receptors leads to enhanced locomotor activity (Van Rossum & Hurkmans, 1964) and dopamine stimulates striatal adenyl cyclase preparations (Kebabian, Petzold & Greengard, 1972; Miller, Horn, Iversen & Pinder, 1974). This work examines the interrelationship between the actions of three potential dopamine receptor stimulants upon both locomotion and cyclic AMP levels in the caudate nucleus of rats.

Drugs dissolved in 5µl of 0.9% NaCl were injected into the lateral ventricles of male Wistar rats using the method of Noble, Wurtman & Axelrod (1967). Twenty minutes later individual animals were placed in an Animex type DSE activity meter and large movements recorded on sensitivity 10 for 24 hours. Cyclic AMP was measured in the striatum and cortex by the *in vitro* system of Munday, Poat & Woodruff (1974). The cyclic AMP was extracted and estimated using a bovine muscle protein (Gilman, 1970). Injections of 2-amino-6, 7-dihydroxy-1,2,3,4-tetrahydronapthalene (ADTN) caused a stimulation of motor activity. There was a lag of 1-2 h before the onset of the effect and once initiated the stimulation continued for up to 18 hours.

The activity characteristically consisted of forward walking, rearing and running movements. Activity was increased to 260% of controls after a 50µg injection and a maximum stimulation of 648% occurred at 200 µg (6 rats in each group). The dimethylether of ADTN injected over a similar dose range was without effect. Ergometrine, 50 or 100 µg, also stimulated locomotor activity, although rearing movements were absent. The time course of the effect was also different, the lag period being short (10-20 min) and the duration 5 hours. The locomotor stimulation induced by either ADTN or ergometrine was abolished by pimozide (0 1 mg/kg i.p.) or haloperidol (0.5 mg/kg i.p.) injected 30 min before intraventricular injection.

Dopamine and ADTN (30 µM) caused striatal cyclic AMP levels to rise to 339 ± 97.3% (4) and 269 ± 33.8% (4) of control levels. Basal levels were in the range 13-18 pmoles cAMP/mg protein. Ergometrine (30 µM) caused a smaller but highly significant stimulation (160 ± 14.3% (6)). The dimethylether of ADTN was ineffective even at 100 µM. Dopamine and ADTN were equipotent at 10, 30 and 100 µM which agrees with iontophoretic studies (Woodruff, Elkhawad, Crossman & Walker, 1974). At 30 µM all three compounds were without effect upon cortical cyclic AMP, but at 100 µM dopamine and ergometrine caused an approximate doubling of the levels.

These results support the contention that ergometrine and ADTN stimulate dopamine receptors in the striatum, the dimethylether of ADTN being ineffective. We have presented evidence for the involvement of cyclic AMP in the behavioural response to these drugs.

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Inhibition of dopamine-sensitive adenylate cyclase in rat basal ganglia and other hormone sensitive adenylate cyclase systems by neuroleptic drugs.

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Homogenates of brain areas containing dopaminergic synapses respond to low concentrations of dopamine by increased cyclic AMP production. The action of dopamine is inhibited by neuroleptic drugs, and mimicked by dopamine agonists (Kebabian, Petzold & Greengard, 1972; Miller, Horn, Iversen & Pinder, 1974). Neuroleptics also inhibit other adenylate cyclase systems (Wolff & Jones, 1970). In the present study we have investigated the specificity of inhibition of these various systems and their possible significance to the mode of action of neuroleptics.

Dopamine-sensitive adenylate cyclase in rat striatum was assayed as previously described (Miller *et al*, 1974). Stimulation produced by dopamine (10^{-4}M) in striatal homogenates was inhibited by (+)-butaclamol with an IC50 of 1.8×10^{-7} M. (−)-butaclamol, however, was ineffective. Butaclamol is a neuroleptic of novel structure and is the first to exhibit optical isomerism. Its interaction with the dopamine receptor appears to differ from any other neuroleptic so far described. It is known that the neuroleptic activity of butaclamol resides solely in the (+) isomer. This again illustrates the close correspondence between neuroleptic activity and dopamine receptor blockade.

The inhibition caused by (+)-butaclamol was competitive with dopamine except at high drug concentrations (10^{-5}M). Inhibition of the effect of dopamine by other neuroleptics at concentrations approximately equal to their IC50 values proved also to be competitive. This included drugs of the phenothiazine, butyrophenone and dibenzodiazepine classes such as thioridazine, haloperidol, clozapine and loxapine. Phenothiazines and butyrophenones also both acted within one minute of their addition to the assay system.

Neuroleptic drugs were also tested on other hormone sensitive adenylate cyclase systems such as the β-adrenoceptor linked system in isolated adipocytes and the glucagon-sensitive system in liver plasma membranes. Inhibition of adenylate cyclase in these systems occurs at higher drug concentrations than in the dopamine-sensitive systems. Thus, for example, the IC50 for chlorpromazine in the β-adrenoceptor system was approximately 2×10^{-4}M against a maximally stimulating concentration of 10^{-4}M noradrenaline, whereas the corresponding IC50 for the dopamine-sensitive cyclase was 1×10^{-6}M. Inhibition of the glucagon-sensitive system occurred at chlorpromazine concentrations (10^{-4}M) which did not inhibit basal enzyme activity in liver plasma membranes although at higher drug concentrations basal activity was inhibited. Inhibition of the glucagon-sensitive system by chlorpromazine (5×10^{-4}M) was non-competitive. In this system α and β-flupenthixol were both equally effective at 10^{-4}M, and haloperidol was ineffective at 10^{-4}M. This does not parallel the neuroleptic properties of these compounds. The effects of neuroleptics on the glucagon or noradrenaline-sensitive adenylate cyclases occurred at concentrations at which other membrane bound enzymes are also affected and probably reflect the general membrane stabilizing effects of neuroleptics rather than direct drug/receptor interactions of the type that occur with the dopamine-sensitive system.

Richard Miller is an M.R.C. Scholar.

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Some effects of age upon irreversible inhibition of cardiac MAO

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It has been suggested, from evidence derived from the use of irreversible inhibitors such as clorgyline that the monoamine oxidase (MAO) in many tissues exists in two forms, called 'species A' and 'species B' (Johnston, 1968). At present, it is not clear whether these two species of enzyme represent differences in localization, e.g., intra- and extraneuronal MAO (Goridis & Neff, 1971), or differential binding of membrane lipid to the same enzyme protein (Tipton, Houslay & Garrett, 1973). In the rat heart, tyramine is metabolized solely by species A while benzylamine is metabolized by both A and B (Lyles & Callingham, 1974). With this latter substrate, the pattern of inhibition produced by clorgyline depends upon the age of the animal.

Hearts were removed from male Wistar rats and male CFLP mice and homogenized in 0.001 M potassium phosphate buffer at pH 7.4. Assay of MAO activity involved the use of ^{3}H-tyramine and ^{14}C-benzylamine as substrates. Inhibitor concentrations varied from 5×10^{-11} to 5×10^{-4} M.

In rats of mean body weight 151 g, benzylamine oxidation produced a double sigmoid inhibition curve with clorgyline. The plateau region of this curve, which occurred at inhibitor concentrations of 5×10^{-8} to 5×10^{-7}M, suggested that species A represented about 35% of the total enzyme activity. In younger rats of body weight 63 g, the plateau shifted to indicate a smaller (23%) proportion of species A. In rats weighing 36 g, the inhibition curve was essentially a single sigmoid typical of species B. However, in older rats weighing 317 g and 414 g, the plateau shifted in the opposite direction suggesting an increased proportion of species A (50% and 70%, respectively). Confirmation of this was obtained using the MAO inhibitor deprenyl. This compound while producing similar inhibition curves to clorgyline, is more active on species B. Benzylamine oxidation again resulted in a double sigmoid inhibition curve, and the plateau region also shifted to indicate an increase in the proportion and amount of species A with increase in the age of the rat. On this evidence, it is suggested that the increase in the specific activity of rat heart MAO seen in the growing rat is mediated largely by an increase in the amount of species A.

In the adult mouse heart, the inhibition curves using clorgyline were different from those in the rat. Using tyramine as substrate, a double sigmoid curve was found, with species A representing about 20% of the total activity. However, benzylamine produced a single sigmoid curve characteristic of species B.

It would appear that the use of the irreversible MAO inhibitors, clorgyline and deprenyl, with a variety of substrates can be used to investigate the possibility of selective changes in the nature of the enzyme that may accompany changes in its specific activity.

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Tricyclic antidepressants and monoamines: the relationship between uptake blockade and potentiation of neuronal responses

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It has previously been reported that the tricyclic antidepressants imipramine and desipramine can potentiate the responses of single brain cells to micro-electrophoretically applied monoamines (Bradshaw, Roberts & Szabadi, 1974) The potentiating effect of the antidepressants is generally ascribed to the efficacy of these drugs in blocking the uptake of monoamines into nervous tissue (Schildkraut, 1965). In order to investigate this hypothesis we have examined whether the tricyclic antidepressants can potentiate neuronal responses to the monamines in situations where uptake mechanisms are unlikely to be involved.

Single spontaneously active neurones were studied in the cerebral cortex and corpus striatum of the halothane-anaesthetized rat. All the drugs were applied by microelectrophoresis. Repeated responses to monoamines were compared following a brief application of an antidepressant (desipramine or iprindole).

1. It has been reported that iprindole is ineffective in blocking the uptake of noradrenaline (NA), 5-hydroxytryptamine (5-HT) and dopamine (DA) into brain tissue (Ross, Renyi & Ögren, 1971). We have found, however, that responses of single neurones to NA, 5-HT and DA can be potentiated by iprindole (18 cells in the cerebral cortex, 6 cells in the corpus striatum).

2. Although desipramine is a powerful inhibitor of NA uptake, its effects on the uptake of DA are very weak (Shore, 1972). We have found that responses of single striatal neurones to DA are potentiated by desipramine (7 cells).

3. In previous experiments we have found that cortical neurones can respond to microelectrophoretic application of mescaline, and that these responses are pharmacologically similar to those evoked by NA and 5-HT (Bevan, Bradshaw, Roberts & Szabadi, 1974). It has been reported that mescaline has an extremely low affinity for NA uptake mechanisms in the periphery (Iversen, 1967). Nevertheless, we have found that responses of cortical neurones to mescaline can be potentiated by desipramine (10 cells).

These findings indicate that uptake blockade cannot fully explain the potentiating effects of the tricyclic antidepressants on neuronal responses to the monoamines. An alternative explanation could be that the potentiation seen in our experiments is due to a post-synaptic effect of the anti-depressants (Bradshaw, Roberts & Szabadi, 1974).

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Effect of drugs on metoclopramide-induced catalepsy and increase in striatal homovanillic acid content

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Metoclopramide disturbs the extrapyramidal control of motor functions in man (Casteels-Van Daele, Jaeken, Van Der Schueren, Zimmerman & Van Den Bon, 1970) and in rats (Costall & Naylor, 1973). In mice metoclopramide-induced catalepsy is associated with a nearly five-fold elevation of striatal homovanillic acid (HVA) content (Ahtee & Buncombe, in press). In the present experiments the mechanism of the cataleptic action of metoclopramide was studied by using drugs, which alter the dopaminergic or cholinergic activity or the content of γ-aminobutyric acid (GABA) in the central nervous system.

Female Wistar rats (180-220 g) were used. Catalepsy was scored by using four tests (3 cm high bar, 9 cm high bar, parallel bars, and vertical grid); each test was scored from 0 to 2. The scores of the four tests were added. The striata of two brains were pooled for the spectrophotofluorimetric estimation of HVA. Metoclopramide and apomorphine were injected s.c., atropine and aminooxyacetic acid (AOAA) i.p.

The dose of 20 mg/kg of metoclopramide induced in rats catalepsy which was maximal at 2-3 h after injection. Apomorphine (10 mg/kg) and atropine (50 mg/kg) significantly reduced the cataleptic effect of metoclopramide. AOAA (25 mg/kg) potentiated the cataleptic effect of 5 mg/kg of metoclopramide. The dose of 20 mg/kg of metoclopramide caused a six-fold elevation of striatal HVA content, but did not alter the brain dopamine content. Apomorphine decreased the striatal HVA content by 66-69% ($p < 0.01$) both in control and in metoclopramide-treated rats. Atropine or AOAA did not alter the metoclopramide-induced elevation of striatal HVA content.

The increased HVA and unchanged dopamine content in the brain of metoclopramide-treated rats suggests that dopamine formation is increased. Thus metoclopramide could act as has been suggested for the neuroleptic compounds (Carlsson & Lindqvist, 1963) by directly blocking the striatal dopaminergic receptors, whereafter a neuronal feedback mechanism could increase the production of dopamine. This assumption is supported by the finding that apomorphine, a drug which is thought to stimulate the dopaminergic receptors, antagonized both the cataleptic and HVA increasing effects of metoclopramide. Moreover, AOAA, a drug which increases the concentration of GABA which is thought to have an inhibitory influence on dopaminergic striatal neurons, potentiated the metoclopramide-catalepsy. However, the mechanism of metoclopramide-catalepsy seems to be somewhat different from the catalepsy induced by neuroleptic compounds, because atropine antagonized the catalepsy but not the HVA increase produced by metoclopramide, whereas atropine is known to antagonize both the catalepsy and HVA increase produced by neuroleptic compounds (Andén & Bédard, 1971; O'Keeffe, Sharman & Vogt, 1970).

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Drug effects on avoidance behaviour in selected strains of rats

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Roman High (RHA) and Roman Low Avoidance (RLA) strain rats were produced by selective breeding, using as selection criteria high or low rates of conditioning in a shuttle-box (Broadhurst & Bignami, 1965). Using conventional bioassay techniques we have found significant differences between the two strains in acetylcholine concentrations in the brain. RLA rats showed significantly higher concentrations than the RHA rats (2.06 ∓ 0.34 μg/g as compared to 1.73 ∓ 0.34 μg/g). When brain regions were examined the differences were most noticeable in the cortex and medulla. No differences were found in acetylcholinesterase activity.

We have also investigated the effects on conditioning behaviour in the two strains of drugs directly or indirectly affecting cholinergic mechanisms. Groups of 10 male RHA, RLA and Porton strain rats weighing 180-200 g were trained in a shuttlebox on 4 consecutive days, 15 min after treatment with either n-ethyl 3-piperidyl benzilate (NEPB, a potent atropine-like drug) 1.0 mg/kg i.p., physostigmine salicylate, 0.12, 0.06 or 0.03 mg/kg s.c. or (+) amphetamine 0.1 mg/kg i.p. NEPB facilitated avoidance conditioning in all 3 strains, but there were significant differences between the strains both in the intensity of effect and in retention of response. RHA and Porton rats showed a similar degree of enhancement, and avoidance responding was maintained when the drug was withheld for a fifth training session. RLA rats showed only slight facilitation of their normally-low responding rate, and when drug was withheld, performance returned to control levels. The rate of conditioning of all 3 strains was decreased by physostigmine, the effect on RLA rats was most marked, these animals actually showing a decreased avoidance with the lowest doses. In all strains (+) amphetamine produced facilitation similar to that seen after NEPB. When rats were given (+) amphetamine and NEPB simultaneously in doses (0.075 mg/kg and 0.75 mg/kg respectively) which alone produced no response, all strains showed facilitated avoidance.

The fact that differences in brain acetylcholine concentrations are found in strains of rats selected to display behavioural differences would support the hypothesis of a central cholinergic system controlling certain types of behaviour (Carlton, 1969) and such a hypothesis is further supported by the differential responses of the strains to NEPB and physostigmine. The observation that amphetamine also produces a differential response, despite the absence of strain differences in synthesis and turnover of catecholamines (Coyle, Wender and Lipsky, 1973) might further suggest that the cholinergic system may be activated by adrenergic neurones.

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A stereospecific action of morphine on brain stem neuronal activity: a microiontophoretic study

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Work in the unanaesthetized rabbit (Herz, Albus, Metys, Schubert & Teschemacher, 1970) suggests that morphine exerts at least part of its antinociceptive effect through an action in the brain stem. Previous work has confirmed that neurones in the brain stem of urethane-anaesthetized rats are sensitive to microiontophoretically applied morphine (Bradley & Dray, 1973; 1974). Furthermore, morphine excites as well as depresses single neurone activity. Subsequent work (Bramwell & Bradley, 1974) revealed that only morphine-induced depression is sensitive to antagonism by naloxone, morphine-induced excitation being unaffected or potentiated.

The present study was undertaken to show whether morphine-induced depression was produced by another narcotic, namely levorphanol, and to see whether its inactive isomer, dextrorphan, was lacking in activity. An attempt was also made at characterizing morphine excitations in terms of the sensitivity of such cells to acetylcholine (ACh) and acetyl β-methylcholine (MeCh).

Male albino rats were prepared as previously described (Bradley & Dray, 1974) under urethane anaesthesia. Drug solutions were as used previously (Bramwell & Bradley, 1974) except for the following: levorphanol tartrate (0.013-0.026 M, pH 4.5 Roche) dextrorphan tartrate (0.013-0.026 M, pH 4.5 Roche); dextrorphan substance (0.013 M, pH 4.5 Roche) acetyl β-methylcholine chloride (0.25 M, pH 5.0 Koch-Light).

Only spontaneously active brain stem neurones were recorded. 5/52 cells tested with levorphanol were depressed, an effect antagonized by naloxone. 4/52 neurones were excited by levorphanol, an effect not antagonized by naloxone. The response of these cells to morphine tended to parallel their response to levorphanol—i e no cell responded to morphine in an opposite way to levorphanol; however, more cells responded to morphine with excitation (12/19).

Although 6/56 cells were depressed by dextrorphan, none of these cells was depressed by morphine, nor were the effects antagonized by naloxone In addition, 4/56 cells were excited by dextrorphan compared with 15/43 cells excited by morphine. In contrast, 12/56 cells tested with dextrorphan exhibited spike height reduction compared with 20/52 for levorphanol and 0/62 for morphine.

As previously reported for NA and 5-HT (Bradley & Dray, 1974) no correlation existed between the response to morphine and those to ACh and MeCh. Once again, however, (Bramwell & Bradley, 1974) a large proportion (14/14) of cells excited by morphine (15/62) were also excited by ACh, but not by MeCh (8/14). If activation of cholinergic receptors is responsible for morphine excitation in the brain stem these are unlikely to be purely muscarinic in nature.

In conclusion, morphine-induced depression of brain stem neuronal firing, which is sensitive to naloxone reversal at low currents and with dilute solutions, appears to represent a stereospecific narcotic action shared by levorphanol but not by dextrorphan. In contrast, morphine-induced excitation represents an action shared by morphine, levorphanol and dextrorphan which is potentiated by naloxone and nalophine.

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Tolerance to sympathomimetic bronchodilators in the guinea pig lungs

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Between 1959 and 1966, in England and Wales an increase in asthma mortality rate was noted which was related to the use of pressurized aerosols containing sympathomimetic bronchodilators. Several suggestions were proposed to explain why pressurized aerosols caused an increase in asthma deaths and these include:

1. Irritation of the respiratory tract exacerbating the asthmatic attack.
2. Pulmonary vasodilator effect of some bronchodilator drugs leading to reduced arterial oxygen tension.
3. Sensitization of the heart to sympathomimetic amines by the propellants of the aerosol.
4. Cardiac stimulatory effect.

Objections have been raised to all these postulates, but more recently Conolly, Davis, Dollery & George (1971) suggested that excessive use of sympathomimetic amines by patients may produce a state of drug-induced cross-resistance to endogenous sympathetic stimulation in the respiratory tract which might lead to a deterioration in asthmatic state.

A simple isolated tissue technique perfusing the bronchial tree through the trachia as described by Tainter, Pedden & James (1934) was utilized to investigate this hypothesis in the guinea pig. A flow recorder was used to determine the rate of perfusion. Pretreatment of the animals subcutaneously three times daily with adrenaline (5 μg/kg) reduced the response of the isolated perfused histamine constricted lungs of the animal when it was challenged with adrenaline (Figure 1). A similar effect was observed when the guinea pigs were pretreated with isoprenaline or adrenaline and challenged with either the same or a different sympathomimetic bronchodilator. The longer the animals were pretreated and the higher the dose of bronchodilator, the greater was the degree of tolerance developed.

Similarly, tolerance developed to aminophylline following pretreatment with aminophylline or isoprenaline, and cross tolerance developed to adrenaline following pretreatment with aminophylline.

These results suggest that asthmatic patients who use bronchodilators excessively may become refractory to these drugs, and support the hypothesis that induced cross-resistance to endogenous sympathetic stimulation could lead to a deterioration in the asthmatic state of these patients.

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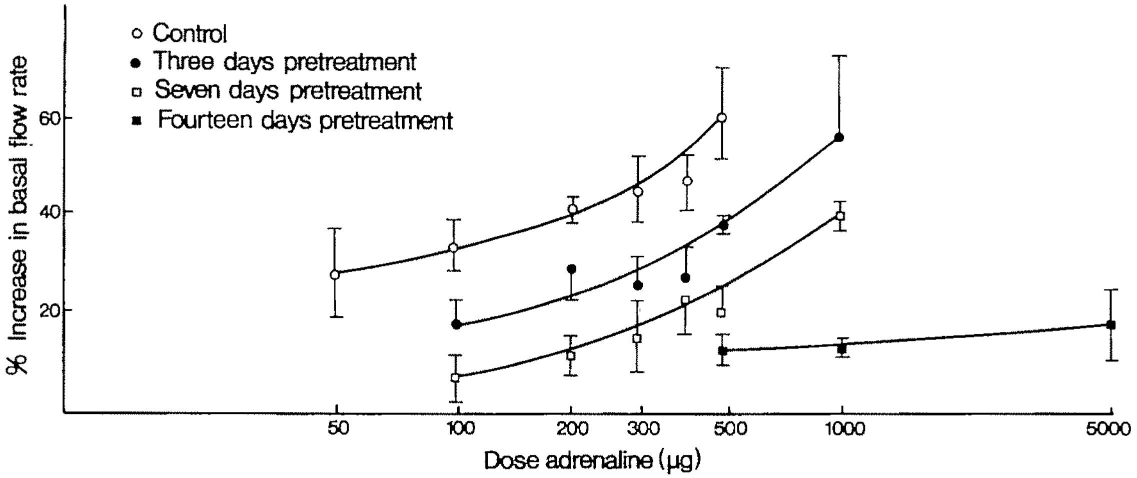

Figure 1 The effect of pre-treatment with 5 μg/kg adrenaline on the response of the guinea-pig isolated perfused histamine-constricted lungs to adrenaline.

Actions of prostaglandins on the arterial system of the sheep: some structure-activity relationships

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Injection of prostaglandin D_2 (PGD_2) into the thoracic aorta of the sheep produces within a few seconds a short lasting rise in systemic arterial blood pressure; the isomeric PGE_2 elicits a fall in blood pressure by this route (Horton & Jones, 1974). A direct action on arterial resistance vessels is indicated for both effects. $PGF_{2\alpha}$ is about 60 times less active than PGD_2 as a pressor agent and $PGF_{2\beta}$ about 150 times less active than PGE_2 as a depressor agent.

Two further observations have been made.
(a) If a sheep is given an intravenous infusion of PGA_1 (0.6-5.0 mg kg^{-1} h^{-1}), a pronounced fall in blood pressure is produced from which there is partial recovery during the infusion. PGE_2 now fails to produce a depressor response whereas PGD_2 and $PGF_{2\alpha}$ are unaffected. Under these conditions $PGF_{2\beta}$ gives a pressor response.
(b) In the pregnant ewe, the depressor response to intra-aortic PGE_2 is attenuated (Horton & Maule Walker, 1974). The sensitivity to the pressor actions of PGD_2 and $PGF_{2\alpha}$ is maintained however. $PGF_{2\beta}$ produces a biphasic response, pressor to depressor, which can be mimicked by injecting an appropriate mixture of PGD_2 and PGE_2.

These results indicate that a particular prostaglandin can act on two systems in the peripheral vasculature to cause vasconstriction and vasodilatation, the resultant effect being dependent on its relative potencies on the two systems and the relative sensitivities of the systems.

A number of other prostaglandins have been investigated and two major structure-activity relationships have emerged.

(i) Oxidation of the 15(S)-hydroxyl to a ketone results in loss of depressor activity. Thus 15-oxo PGE_1 elicits no change in blood pressure at doses 100-300 times the dose of PGE_1 producing a fall of 10 mm Hg. In contrast, pressor activity is either unaffected or enhanced and 15-oxo $PGF_{2\alpha}$ is 5-10 times more active than $PGF_{2\alpha}$. 15-oxo $PGF_{2\beta}$ is a pressor agent of low potency.
(ii) Saturation of the 5,6-*cis* and 13,14-*trans* double bonds results in marked loss of pressor activity. Thus PGD_2 and 15-oxo $PGF_{2\alpha}$ are about 8 times more active than PGD_1 and 15-oxo $PGF_{1\alpha}$ respectively and about 100 times more active than 13,14-dihydro PGD_1 and 13,14-dihydro-15-oxo $PGF_{1\alpha}$ respectively. Depressor activity is little affected by these changes and the potencies of the 2-, 1-, and 13,14-dihydro-1-series analogues with either the E, 11-deoxy E, A or B ring structures differ by less than a factor of three.

Of the compounds tested it is suggested that 15-oxo $PGF_{2\alpha}$ shows the highest specificity for the pressor system, and 13,14-dihydro PGE_1 the highest specificity for the depressor system.

Further studies will be concerned with the identification of systems in other tissues showing similar agonist structure-activity relationships. In this connexion, the recent report by Dawson, Lewis, McMahon & Sweatman (1974) showing that $PGF_{2\alpha}$, 15-oxo $PGF_{2\alpha}$ and PGD_2 are of the same order of potency as bronchoconstrictor agents is of great interest.

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Release of prostaglandins and incapacitation after injection of endotoxin in the knee joint of the dog.

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Ferreira, Moncada & Vane (1974) suggested that incapacitation during inflammation in the knee joint of the dog is due to sensitization of pain receptors by locally released prostaglandins. Since injection of endotoxin into the knee joint cavity induces incapacitation (Morgan & Bennett, 1947; Van Arman, Carlson, Kling, Allen & Bondi, 1974) and since endotoxin releases prostaglandins (Collier, Herman & Vane, 1973, Herman & Vane, 1974), we decided to study the time course of this inflammatory process, the release of prostaglandins during its development and the effect of local treatment with indomethacin.

Mongrel dogs of either sex were anaesthetized with thiopentone (10 mg/kg intravenously). Endotoxin (*E. coli* Difco 0111B4,) 25 ng/kg in 0.5 ml of sterile saline, was injected in one of the knee joint cavities and sterile saline (0.5 ml) was injected in the contralateral joint. Symptoms of incapacitation ranging from limping, occasional 3-legged gait to complete 3-legged gait were scored. One hour to 1½ h after the injection, symptoms started to develop and were most pronounced from the 3rd to the 5th or 6th hour. In the next 3 to 4 h a gradual recovery occurred. The symptoms had disappeared completely within 24 hours.

At different times after the injection of endotoxin, synovial fluid was sampled from both joints under light thiopentone anaesthesia and the cavities washed twice with 2 ml of sterile saline. Total samples were extracted and bioassayed for PG-like activity (Gilmore, Vane & Wyllie, 1968). The results show a close relationship between the prostaglandin content of the samples and incapacitation: synovial fluid obtained before injection of endotoxin had no PG-like activity; the mean PG-like activity of the samples collected just before limping started and during maximal symptoms was respectively 43 (range: 0-191; 10 experiments) and 123.5 (range: 11-141; 7 experiments) ng PG E_2-equivalents. Only in 1 out of 10 dogs PG-like activity could be detected in synovial fluid obtained after 24 h when symptoms had disappeared. No PG-like activity was found in the saline treated joints.

In 9 dogs endotoxin was injected together with indomethacin (200 μg/kg dissolved in phosphate buffer 0.1 M, pH 8.9). In 7 of these dogs no symptoms developed up to 7 h after the injection. In 2 of them, mild symptoms appeared. No PG-like activity was found in the dogs treated with indomethacin in which no symptoms developed; in 2 dogs with symptoms, small amounts of PG-like activity could be detected.

These experiments show that endotoxin injected in the cavity of the knee joint produces incapacitation which is closely related to the appearance of prostaglandins in the joint fluid and which is susceptible to local treatment with indomethacin. These findings are consistent with the hypothesis (Ferreira, 1972; Ferreira, *et al.*, 1973, 1974) that prostaglandins are important in the development of pain (possibly by sensitizing the pain receptors) and that aspirin-like drugs exert their analgesic action by the abolition of the sensitization of the pain receptors caused by the local release of prostaglandins.

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The protective effect of sodium meclofenamate in experimental endotoxin shock

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The administration of *E. coli* endotoxin to anaesthetized cats results, within 1-3 min, in a rise in pulmonary artery pressure and in transient decreases in systemic arterial blood pressure and in myocardial contractility. The secondary shock phase is characterized by systemic hypotension, a reduced stroke volume and a severe metabolic acidosis (Parratt, 1973). Only 6% of animals survive longer than 6 h after the intravenous administration of 2 mg/kg of endotoxin. Pretreatment with indomethacin not only abolishes the initial pulmonary hypertension but also delays the onset of the secondary shock phase and increases survival (Parratt & Sturgess, 1974). One possible explanation for these protective effects is inhibition of prostaglandin synthesis and the purpose of the present experiments was to determine the effectiveness of sodium meclofenamate, which is a more active inhibitor of prostaglandin synthetase than indomethacin (Flower, 1974) and which has less marked direct effects on cardiac function.

Sodium meclofenamate (0.5, 2.0 and 5.0 mg/kg, intravenously, when administered up to 6 h before the endotoxin) prevented, or markedly reduced, endotoxin-induced pulmonary hypertension and decreases in pulmonary compliance. In control cats, endotoxin (2 mg/kg, intravenously) increased pulmonary artery pressure from 19 ± 1 mmHg (systolic) and 10 ± 1 mmHg (diastolic) to 41 ± 7 and 27 ± 5 mmHg respectively. The corresponding pulmonary artery pressures in the cats pretreated with sodium meclofenamate (2 mg/kg, 30 min before the endotoxin administration) were, pre-endotoxin 22 ± 2 mmHg (systolic) and 11 ± 1 mmHg (diastolic) and, 3 min after endotoxin, 25 ± 2 and 15 ± 2 mmHg. This dose of sodium meclofenamate also prevented, or delayed, the onset of the shock phase. In control animals the diastolic blood pressure 3 h after endotoxin was a mean of 25 ± 12 mmHg below the mean pre-endotoxin level of 83 ± 5 mmHg. This decrease in diastolic pressure after endotoxin was not observed in the animals pretreated with sodium meclofenamate and 29% of these cats were alive at 6 h (compared with only 6% administered endotoxin alone).

The effects of sodium meclofenamate (2.0 mg/kg) were also examined when administered 30 min after the injection of endotoxin and every 1 h thereafter. This treatment was associated with marked elevations in systemic blood pressure during the shock phase and with increased survival (40% at 6 h).

These experiments support the concept that the release of a prostaglandin (probably $F_{2\alpha}$) mediates the initial pulmonary vasoconstriction that results from endotoxin administration in cats and provides evidence that inhibiting prostaglandin release during the shock phase has beneficial haemodynamic consequences which increase the possibility of survival.

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Inhibition of the pulmonary inactivation of prostaglandins in rabbit *in vivo*

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Prostaglandins (PGs) of the E and F series are efficiently inactivated in the pulmonary circulation of several species *in vivo* (Ferreira & Vane, 1967) and of guinea-pig *in vitro* (Piper, Vane & Wyllie, 1970). This inactivation can be strongly inhibited *in vitro* by di-4-phloretin phosphate (DPP) at concentrations well below those causing antagonism of PG actions (Crutchley & Piper, 1973, 1974). The present study investigates whether DPP inhibits pulmonary inactivation of PGE$_2$ by rabbits *in vivo*.

Male Dutch rabbits 1.8 to 2.5 kg were anaesthetized by pentobarbitone sodium 40 mg/kg i.v. A polyethylene cannula was introduced retrogradely into the aortic arch via the right carotid artery for i.a. infusions. A similar cannula for i.v. infusion was introduced into the superior vena cava via the right jugular vein. Blood pressure was recorded from the left carotid artery or a femoral artery. Saline, or DPP (in solution, in saline) was infused continuously into the left jugular vein. PGE$_2$ infusions of 1.5 min duration were given i.a. or i.v. and the resulting falls in blood pressure measured. Control and test measurements were taken in the same animals.

PGE$_2$ was much more potent via the i.a. route, the difference between i.a. and i.v. responses being taken as a measure of pulmonary inactivation. DPP 25 to 100 μg kg^{-1} min^{-1} potentiated the i.v. responses and shifted the dose responses curve for i.v. PGE$_2$ to the left, to approximate to that for i.a. doses ($n = 18$). DPP had no effect on the i.a. responses, suggesting that potentiation of i.v. effects was due to decreased inactivation of PGE$_2$ in the lung. Potentiation of i.a. responses by DPP was seen, however, with longer infusions of PGE$_2$ (6 to 8 min). This was presumably due to the cumulative effects of circulating i.a. doses. No direct antagonism of PGE$_2$ was seen.

DPP 25 to 100 μg kg^{-1} min^{-1} also potentiated the depressor actions of i.v. infusions of PGF$_{2\alpha}$ and the increase in gastrointestinal motility due to i.v. infusions and injections of PGs E$_2$ and F$_{2\alpha}$ ($n = 5$). This was presumably due to impaired pulmonary PG inactivation.

The percentage inhibition of pulmonary inactivation by these doses of DPP was 75% at maximum but this caused marked potentiation of PG effects, emphasizing the importance of pulmonary inactivation of PGs.

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Factors influencing the turnover of prostaglandin synthetase

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Evidence is accumulating that prostaglandin generation is of physiological and pathological importance, so the mechanism(s) by which intracellular levels of prostaglandin synthetase are regulated are clearly of interest. Continuous *de novo* synthesis of enzymes is a property of all viable cells and one method by which the cellular concentration of certain types of enzymes can be rapidly regulated (in response to hormone stimulation for example) is by changes in their rate of synthesis or destruction. Lands, LeTellier, Rome & Vanderhoek (1973) have shown that *in vitro* prostaglandin synthetase catalyses its own destruction during substrate oxygenation. If such 'self-destruction' occurs *in vivo*, a tissue such as the kidney which has a high biosynthetic capacity (Somova, 1973) should demonstrate continuous synthesis of the enzyme also.

To test this possibility we injected (i.p.) four male rats (200-250 g) with 4 mg cycloheximide

Table 1 Effect of cycloheximide on PG biosynthesis and *de novo* protein synthesis*

Time (hr)	*p moles ^{14}C-lysine incorporated into protein*	*p moles ^{14}C-PGs synthesized*	*p moles 15-keto PGE$_2$ synthesized*
0	65.32	7.8	58.9
1	50.34	8.9	33.0
2	23.19	10.7	14.1
3	21.64	12.2	16.4
4	15.34	10.9	14.4
5	12.93	11.0	13.9
6	8.08	10.4	14.1
7	6.96	10.7	11.3

* Each figure is the mean of two results

(an inhibitor of protein synthesis). Three hours later the rats were killed and the kidneys were removed. Prostaglandin biosynthesis in kidney homogenates was measured by bioassay (see Gilmore, Vane & Wyllie, 1968) after 10 min incubation at 37° C with 20 μg added arachidonic acid. Protein synthesis was quantitated in slices of kidney by measuring the incorporation of (0.1 μc) ^{14}C-lysine into protein during 1 h in a shaking water bath at 37° C. The mean prostaglandin biosynthesis in the two control animals was 82.2 p moles/kidney. Surprisingly in rats treated with cycloheximide prostaglandin production was 428.4 p moles/kidney, even though the protein synthesis was inhibited by 63.7%. One reason for this apparent stimulation of synthesis could be that the prostaglandin metabolizing enzymes were being blocked. To check this possibility, 14 male rats were injected with 4 mg cycloheximide. Each hour, thereafter, two of the rats were killed and the kidneys removed. Prostaglandin biosynthesis here was measured by the conversion of ^{14}C-arachidonic acid to labelled PGE$_2$ and F$_{2\alpha}$, which were extracted from the aqueous homogenates and isolated by t.l.c. after 10 min incubation in a shaking water-bath at 37° C. Unlabelled prostaglandin E$_2$ (10 μg) was added to homogenates to protect labelled prostaglandins from destruction by metabolizing enzymes. Metabolism of the prostaglandins was measured by the conversion of (0.01 μCi) ^{3}H-PGE$_2$ to its 15-keto derivative, which was extracted from aqueous homogenates after 10 min incubation at 37° C and isolated by t.l.c. Protein synthesis was measured as before.

Table 1 clearly shows that although the capacity of the kidneys to synthetize prostaglandins is undiminished even when protein synthesis was reduced by 80-90%, the capacity of the kidney to metabolize prostaglandin was greatly reduced. A preliminary study excluded the possibility that this was a direct blocking action of the drug.

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Inhibitory effect of L-ascorbic acid on the yield of prostaglandin F from the guinea-pig uterine homogenates

D.M. PUGH, S.C. SHARMA* &
C.W.M. WILSON

Department of Pharmacology, University of Dublin, Ireland

Poyser (1972) demonstrated that homogenized guinea-pig uterus has the ability to biosynthesize prostaglandins from endogenous precursors and that this process can be inhibited by indomethacin. Earlier studies (van Dorp, 1971) have shown that certain antioxidants, such as α-tocopherol and propylgallate, have inhibitory effects on the biosynthesis of prostaglandins. The experiments here reported were carried out to determine whether L-ascorbic acid (L-AA), which forms an oxidation-reduction system in the body, would have an effect on the *in vitro* yield of prostaglandin F from guinea-pig uterine homogenate.

Guinea-pigs fed on the normal diet were killed on day 14 of the oestrous cycle and the uterii were removed. Each uterus was freed of fat and blood and homogenized mechanically in ice-cold Krebs solution for 10 minutes. More Krebs solution was added to give a final tissue concentration of 100 mg/ml. In each experiment, tissue from one animal was incubated with a range of L-AA concentrations. Four tubes were set up for each concentration of L-AA and for tissue samples to which no L-AA was added. All replicates contained 200 μl of homogenate to which 25 μl volumes of either Krebs solution or L-AA in Krebs solution were added. In the latter case, the concentration of L-AA was designed to yield the final concentration of ascorbic acid shown in the table. All the tubes were incubated for one hour at 37° C. To the tubes in Group A, nine volumes of 96% ethanol were added prior to incubation while to all other tubes ethanol was added at the end of incubation so as to terminate prostaglandin synthesis (Ånggård & Samuelsson, 1965). From all incubated tubes PGF was extracted and assayed as described earlier (Sharma, Hibbard, Hamlett & Fitzpatrick, 1973). The effect of L-AA additions on pH was determined before but not after incubation.

The results shown in the table indicate that additions of varying concentrations of L-AA have an inhibitory effect on the yield of PGF from the uterine tissue homogenates. Although an ascorbic acid-dependent effect seems to be lacking at higher concentrations, there is a possibility that such relationship does exist at lower concentrations. However, since in other tissues prostaglandin inhibition appears to be pH-dependent (Rose & Collins, 1974) and since the number of observations for the lower concentrations of ascorbic acid in the present investigation is limited, further work would be required to clarify this point.

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Table 1 Prostaglandin F content of guinea-pig uterine homogenate ng/100 mg tissue

Groups	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*
L-ascorbic acid (μg/ml homogenate)	–	–	6.25	12.5	25	50	125	625	1250
pH before incubation	7.42	7.42	7.41	7.40	7.37	7.32	7.17	5.93	4.51
PGF content (mean ± s.e.)	31.6± 4.3 (n = 8)	119.5± 10.5 (n = 8)	97.6± 9.4 (n = 3)	84.1± 7.9 (n = 3)	70.3± 8.7 (n = 8)	63.6± 10.2 (n = 3)	67.3± 3.9 (n = 8)	91.8± 2.8 (n = 5)	67.9± 1.5 (n = 5)
Level of significance from Group B	<0.01	–	>0.05	<0.05	<0.01	<0.01	<0.01	<0.05	<0.01

Action of a series of non-steroid and steroid anti-inflammatory drugs on prostaglandin synthesis by the microsomal fraction of rat skin

M.W. GREAVES, W.P. KINGSTON* & KATHLEEN PRETTY

University Department of Dermatology, Newcastle upon Tyne

We have obtained a prostaglandin synthetase-rich microsomal fraction from skin and we report the action of non-steroid and steroid anti-inflammatory drugs on prostaglandin synthesis by this preparation

Freshly excised rat skin was homogenized in tris buffer pH 8.2 (0.2 M). The microsomal pellet was obtained by differential ultracentrifugation and indentified by electron microscopy and measurement of the marker enzyme glucose-6-phosphatase. Prostaglandin biosynthesis by the microsomal fraction was measured by both the radiochemical method and the adrenochrome spectrophotometric method of Takeguchi and Sih (1972).

For the radiochemical method the enzyme preparation was incubated for 20 min at 37°C with the following: arachidonic 0.2 mM: ^{3}H-arachidonic acid, 0.5 μCi, glutathione 0.65 mM; hydroquinone 0.275 mM and bovine serum albumin 10 mg. Prostaglandins E_2 and $F_{2\alpha}$ were extracted from the incubation mixture and identified by the thin-layer chromatographic method of Greaves & McDonald-Gibson (1972a). The reaction mixture in the adrenochrome assay contained arachidonic acid 0.2 mM, adrenaline tartrate 1.0 mM, tris buffer 8.2 (0.2 M). These reagents were pre-incubated for 5 min at 37°C, the reaction being initiated by addition of the pre-incubated mixture to the enzyme preparation. The change in absorbency at 480 nm was followed for 5 minutes. Control reactions with trichloracetic acid inactivated enzyme preparations were carried out in all experiments. The results are shown in Table 1. Inhibition was dose related and the results for the two assay methods agreed closely. Indomethacin, flufenamic acid and mefenamic acid were the most potent inhibitors of synthesis, although the concentrations required for inhibition were high (Vane, 1971; Ziboh, 1973). Five potent corticosteroid agents did not inhibit synthesis over a wide dose range. The corticosteroid results contrast with findings of an earlier study, using a crude skin homogenate as a source of prostaglandin synthetase activity, in which dose-related inhibition by fluocinolone acetonide was demonstrated (Greaves & McDonald-Gibson, 1972b). The results could be explained by the presence in the homogenates, but not in the microsomal fraction, of a factor which facilitates or potentiates an inhibitory action of corticosteroids.

TABLE 1 Inhibition of prostaglandin synthetase by anti-inflammatory drugs

	ID 50 (mM)	
Drug	*Adrenochrome Spectrophotometric assay*	*Radiochemical assay*
Indomethacin	0.75	0.61
Mefanamic	1.5	1.025
Flufenamic acid	1.8	1.27
Naproxen	9.0	*
Ibuprofen	13.5	*
Aspirin	14.0	10.05

* Not assayed

Paracetamol, Hydrocortisone, Dexamethasone, Betamethasone, Triamcinolone acetonide, Fluocinolone acetonide did not inhibit synthesis measured by either assay method over a wide dose range.

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British Journal of Pharmacology

VOLUME 53 NUMBER 3 MARCH 1975

page

Systematic Pharmacology

317 CHAWLA, NARESH, JOHRI, M.B.L., SAXENA, P.N & SINGHAL. K.C. Cholinergic mechanism in central thermoregulation in pigeons

Autopharmacology

323 JONES, MARGARET E.L. & SPRIGGS, T.L.B Noradrenaline and motor transmission in the vas deferens of the mouse

333 MINATOYA, H. & SPILKER, B.A. Lack of cardiac or bronchodilator tachyphylaxis to isoprenaline in the dog

341 BURDEN, D.T. & PARKES, M.W. An investigation of the tachycardia produced by intracerebroventricular injections of isoprenaline in mice

349 SLOTKIN, T.A. Effects of reserpine on the adrenal medulla of the spontaneously hypertensive rat

357 RUBIN, R.P. & WARNER, W. Nicotine-induced stimulation, of steroidogenesis in adrenocortical cells of the cat

Drug Mechanisms

363 ROBINSON, D.A., TAYLOR, J.G. & YOUNG, J.M. The irreversible binding of acetylcholine mustard to muscarinic receptors in intestinal smooth muscle of the guinea-pig

371 HUGHES, J., KOSTERLITZ, H.W. & LESLIE, FRANCES M. Effect of morphine on adrenergic transmission in the mouse vas deferens. Assessment of agonist and antagonist potencies of narcotic analgesics

383 COX, B.M., GINSBURG, M., WILLIS, JULIA & DAVIES, J.K. The offset of morphine tolerance in rats and mice

393 NESHEIM, BRITT-INGJERD. Action of β-adrenoceptor antagonists on the response to isoprenaline in the oestrogen dominated rabbit uterus

403 NESHEIM, BRITT-INGJERD, OSNES, J.-B. & ØYE, I. Role of cyclic adenosine 3′,5′-monophosphate in the isoprenaline-induced relaxation of the oestrogen dominated rabbit uterus

Miscellaneous

409 EVANS, D.P. & THOMSON, D.S. Inhibition of immediate hypersensitivity reactions in laboratory animals by a phenanthroline salt (ICI 74,917)

419 SHORT COMMUNICATIONS

431P PROCEEDINGS OF THE BRITISH PHARMACOLOGICAL SOCIETY, London, 8-10 January, 1975.

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VOLUME 53(4) APRIL 1975

British Journal of Pharmacology

page

473 SNYDER, S.H. (work done in collaboration with Anne B. Young). Fifth Gaddum Memorial Lecture, University of Bristol, September 1974. The glycine synaptic receptor in the mammalian central nervous system (AP1, DM2, SP2)

Systematic Pharmacology

485 PARRATT, J.R. & STURGESS, R.M. *E. coli* endotoxin shock in the cat; treatment with indomethacin (SP1)
489 JOHNSON, K.G. Thermoregulatory changes induced by cholinomimetic substances introduced into the cerebral ventricles of sheep . (SP2, AP1)
499 DE MICHELI, P. & GLÄSSER, A.H. The effects of catecholamines and adrenoceptor blocking drugs on the canine peripheral lymph flow (SP3, AP1, DM2)

Autopharmacology

505 HENDERSON, G., HUGHES, J. & KOSTERLITZ, H.W. The effects of morphine on the release of noradrenaline from the cat isolated nictitating membrane and the guinea-pig ileum myenteric plexus-longitudinal muscle preparation (AP1, DM2, PK3)
513 PACHA, W., SALZMANN, R. & SCHOLTYSIK, G. Inhibitory effects of clonidine and BS 100-141 on responses to sympathetic nerve stimulation in cats and rabbits (AP1)
517 DAWES, P.M. & FAULKNER, D.C. The effect of propranolol on vascular responses to sympathetic nerve stimulation (AP1, DM2, SP1)
525 HOOD, A.J.C., SMY, J.R. & WEETMAN, D.F. An indirect sympathomimetic effect of burimamide on kitten isolated atria (AP1, AP2, SP1)
531 CHAMALES, M.H., GOURLEY, R.D. & WILLIAMS, BETTY J. Effect of acetylcholine on changes in contractility, heart rate and phosphorylase activity produced by isoprenaline, salbutamol and aminophylline in the perfused guinea-pig heart (AP1, DM2, SP1)
539 HIGGS, G.A., McCALL, ELAINE & YOULTEN, L.J.F. A chemotactic role for prostaglandins released from polymorphonuclear leucocytes during phagocytosis (AP2)
547 BEDWANI, J.R. & MARLEY, P.B. Enhanced inactivation of prostaglandin E_2 by the rabbit lung during pregnancy or progesterone treatment (AP2, AP3)
555 HODGES, J.R. & VELLUCCI, SANDRA V. The effect of reserpine on hypothalamo-pituitary-adrenocortical function in the rat (AP3)

Pharmacokinetics

563 BUCZKO, W., DE GAETANO, G. & GARATTINI, S. Effect of fenfluramine on 5-hydroxytryptamine uptake and release by rat blood platelets (PK3, DM1)

569 FANTOZZI, R., FRANCONI, FLAVIA, MANNAIONI, P.F., MASINI, EMANUELA & MORONI, F. Interaction of histamine H_1- and H_2-receptor antagonists with histamine uptake and metabolism by guinea-pig isolated atrium and mouse neoplastic mast cells *in vitro* (PK2, PK3, DM2)

Drug Mechanisms

575 SPEDDING, M., SWEETMAN, A.J. & WEETMAN, D.F. Antagonism of adenosine 5′-triphosphate-induced relaxation by 2-2′-pyridylisatogen in the taenia of guinea-pig caecum (DM1, DM2)

585 KENNEDY, I. & LEVY, G.P. Combined α- and β-adrenoceptor blocking drug AH 5158: further studies on α-adrenoceptor blockade in anaesthetized animals (DM2)

SHORT COMMUNICATIONS

593 el-AZHARY, ROKEA A., KHAYYAL, M.T. & SALEH, SAMIRA. Effect of fluphenazine on tissue noradrenaline concentrations and its interaction with pargyline (SP2, PP, PK2)

596 GREENBERG, R. Indomethacin potentiation of the response of the rabbit portal vein to electrical stimulation: effect of stimulus frequency and train length (AP1, AP2)

598 LARGE, W.A. Effect of tetramethylenedisulphotetramine on the membrane conductance increase produced by γ-amino-butyric acid at the crab neuromuscular junction (DM1)

Systematic Pharmacology

SP1	Cardiovascular system
SP2	Central nervous system
SP3	Other physiological systems

Pharmacokinetics

PK1	Storage, distribution, fate
PK2	Synthesis, metabolism
PK3	Uptake, release

Autopharmacology

AP1	Adrenergic, cholinergic transmission
AP2	Histamine, kinins, prostaglandins, etc.
AP3	Hormones

Drug Mechanisms

DM1	Molecular, cellular, biochemical, biophysical
DM2	Receptors, antagonism, potentiation, tolerance

CP	**Clinical Pharmacology, Experimental Therapy**
CT	**Chemotherapy**
M	**Miscellaneous**
PM	**Pharmacometrics**
PP	**Psychopharmacology**
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Br. J. Pharmac. (1975), 53, 473–484

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THE GLYCINE SYNAPTIC RECEPTOR IN THE MAMMALIAN CENTRAL NERVOUS SYSTEM

S.H. SNYDER (work done in collaboration with ANNE B. YOUNG)

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Sir John Gaddum had a wide-ranging interest in all neurotransmitter candidates known in his time and made important contributions with respect to most of them. Even with his broad vision, Sir John might well have been amazed at the proliferation of new and unexpected transmitter candidates in the central nervous system. Through indirect pharmacological approaches, Gaddum attained considerable insight into receptor mechanisms for drugs and neurotransmitter. Today I will focus on one 'new' central nervous transmitter, glycine, and upon direct biochemical studies of its postsynaptic receptor.

In the mammalian central nervous system the most recognized neurotransmitters are acetylcholine, the catecholamines, noradrenaline and dopamine, and the indole amine, 5-hydroxytryptamine. Intensive studies of the synthesis, release and synaptic inactivation of the catecholamines have helped elucidate the mechanism of action of important drugs which affect behaviour, such as antidepressants, amphetamines, and phenothiazine tranquilizers (Carlsson, Kehr, Lindquist, Magnusson & Atack 1972; Schildkraut, 1973; Snyder, Banerjee, Yamamura & Greenberg, 1974). Similar investigation of the disposition of 5-hydroxytryptamine in the mammalian brain has helped clarify how psychedelic drugs act (Aghajanian, 1972). Yet despite the great amount of interest and clinical relevance of the biogenic amines, quantitatively they are not the major neurotransmitters in the brain and probably account for transmission at only a small percentage of central nervous synapses. Thus it is estimated that noradrenaline is a transmitter of only about 1% of synapses throughout the brain and, even in the hypothalamus, which contains the brain's highest level of noradrenaline only 5% of the nerve terminals contain noradrenaline (Fuxe, Hökfelt, Ritzen & Ungerstedt, 1968; Hökfelt, Jonsson & Lidbrink, 1970).

Dopamine levels in the brain are about the same as those of noradrenaline and in the corpus striatum, where dopamine is most enriched, only about 15% of the nerve terminals contain catecholamines (Hökfelt *et al*, 1970). Autoradiographic studies suggest that even a smaller percentage of neurones in the brain contain 5-hydroxytryptamine (Kuhar & Aghajanian, 1973). Various workers estimate that acetylcholine nerve terminals involve only about 10% of brain synapses (McLennan, 1970).

In quantitative terms, amino acids now appear to be the major neurotransmitters in the mammalian central nervous system. Neurophysiologically, two classes of amino acids are recognized: excitatory amino acids (glutamic and aspartic acids, and cysteic and homocysteic acids) which depolarize most neurones in the mammalian central nervous system, and inhibitory amino acids (γ-aminobutyric acid (GABA), glycine, β-alanine and taurine) which hyperpolarize the neuronal cell membrane (Curtis & Watkins, 1960; Curtis, Hosli & Johnston, 1968a; Curtis, Hosli, Johnston & Johnston, 1968). Of this latter group, GABA and glycine are major candidates for the chemical mediation of inhibitory neurotransmission in the central nervous system. GABA is active throughout the neuroaxis whereas glycine affects neurones only in the spinal cord, brain stem and diencephalon but not in the cerebral cortex.

When brain slices or homogenates are incubated with low concentrations of radioactive GABA or glycine, nerve terminals which presumably utilize either amino acid as their neurotransmitter are selectively labelled by high affinity, sodium-dependent transport systems (Iversen & Neal, 1968; Roberts & Kuriyama, 1968; Iversen & Johnston, 1971; Logan & Snyder, 1972; Bennett, Logan & Snyder, 1972; 1973). Autoradiographic studies of such preparations reveal that the number of GABA accumulating neurones in the brain varies with different regions from about 27% in the cerebral cortex to 45% in the hippocampus, a considerably greater portion of synapses than contain either acetylcholine or the biogenic amines (Fuxe *et al.*, 1968; Hökfelt *et al.*, 1970, Iversen & Bloom, 1972; Iversen & Schon, 1973; Kuhar & Aghajanian, 1973). In the spinal cord, GABA and glycine appear to act at an equal number of synapses. Thus about 25% of spinal cord nerve terminals can be labelled with [^{3}H]-GABA and

25% can be labelled with [^{3}H]-glycine. When slices are exposed to both compounds, 50% of the nerve terminals are labelled (Iversen & Bloom, 1972).

With such an apparently large proportion of inhibitory synapses present, one might expect a comparable number of excitatory connections. What are the major excitatory neurotransmitters? The best candidates are the amino acids, glutamic and aspartic acids (Krnjević & Phillis, 1963; Werman, 1972) and possibly peptides, such as Substance P (Konishi & Otsuka, 1974; Phillis & Limacher, 1974). However, the evidence for specific compounds as excitatory neurotransmitters is less strong than for the inhibitory transmitters.

What criteria should be met for the identification of a neurotransmitter? Universally accepted ones are: (1) the compound is present in the appropriate neurones, (2) it is released when neurones are activated and perhaps most importantly, (3) its synaptic actions closely mimic those of the natural transmitter (Krnjević & Phillis, 1963; Werman, 1966; Werman, 1972). For amino acids, there is little doubt that all are present in neurones in the brain. To study an amino acid neurotransmitter biochemically, however, one must discriminate the 'transmitter pool' from possibly larger pools required for intermediary metabolism and protein synthesis. In the case of glycine, it appears possible to label selectively the neurotransmitter pool by means of the high affinity, sodium-dependent uptake system into 'glycinergic' nerve terminals (Iversen & Neal, 1968; Roberts & Kuriyama, 1968; Iversen & Johnston, 1971; Logan & Snyder, 1972, Bennett *et al.*, 1972; 1973). Nerve terminals which accumulate radiolabelled glycine by the high affinity uptake system can be separated physically from the majority of other nerve terminals by sedimentation of brain homogenates on linear sucrose gradients (Arregui, Logan, Bennett & Snyder, 1972). Glycine accumulated in this way can be released fairly selectively by electrical or potassium-induced depolarization (Bradford, 1970; Hopkin & Neal, 1971; Hamerstad, Murray & Cutler, 1971; Roberts & Mitchell, 1972; Mulder & Snyder, 1974).

In the central nervous system, the uptake and release of neurotransmitters must be analysed in whole tissue preparations rather than at individual synapses, thus making it difficult to fulfil rigorously the first two criteria. In contrast, methods for making intracellular recordings from spinal cord motor neurones have enabled investigators to compare directly the inhibitory synaptic actions of glycine with those of the natural inhibitory transmitter (Werman, Davidoff & Aprison, 1968; ten Bruggencate & Engberg, 1968; Curtis *et al.*, 1968a & b). Both glycine and GABA hyperpolarize spinal cord motor neurones with a reversal potential similar to that of the natural transmitter substance. Glycine and GABA can be discriminated pharmacologically. The inhibitory actions of glycine are antagonized by low concentrations of strychnine, while those of GABA are strychnine-resistant. Further, since strychnine blocks selectively the natural mediator for reciprocal or direct inhibition of spinal motorneurones, it appears probable that glycine is the natural inhibitory transmitter in these pathways (Owen & Sherrington, 1911; Bradley, Easton & Eccles, 1953; Larson, 1969; Curtis, Duggan & Johnston, 1971). Strychnine-sensitive glycine inhibition occurs in the brainstem as well as in the spinal cord and then decreases as one ascends the neuroaxis (Krnjević, Randić & Straughan, 1966, Biscoe & Curtis, 1967; Kelly & Krnjević, 1969; Larson, 1969; Curtis *et al.*, 1971; Tebecis & DiMaria, 1972). Interestingly, this regional distribution coincides with the regional distribution of endogenous glycine and of the high affinity neuronal uptake system for glycine (Table 1) (Iversen & Neal, 1968; Roberts & Kuriyama, 1968; Aprison, Shank & Davidoff, 1969a, Aprison, Shank, Davidoff & Werman, 1969b; Iversen & Johnston, 1971; Logan & Snyder, 1972; Bennett *et al.*, 1972; 1973).

The consensus is that glycine is the transmitter of certain small, inhibitory neurones in the brainstem and spinal cord and perhaps of some neurones in higher centres. Within the spinal cord, glycine hyperpolarizes motor neurones and decreases their frequency of firing (Werman *et al.*, 1968; ten Bruggencate & Engberg, 1968; Curtis *et al.*, 1968a & b). These inhibitory interneurones are apparently involved in the coordination of antagonistic muscle groups (Owen & Sherrington, 1911; Bradley *et al.*, 1953; Larson, 1969; Curtis *et al.*, 1971; Eccles, 1972). Thus when a monosynaptic excitatory reflex to extensor motor neurones is stimulated, the activity of the antagonistic flexor motorneurones must be suppressed. In this case, collaterals from the primary afferent inputs to the extensor motorneurones excite inhibitory interneurones (glycine interneurones) which in turn synapse on and hyperpolarize flexor motorneurones, thus inhibiting contraction of the flexor muscles. Strychnine, as an antagonist of glycine, abolishes this reciprocal inhibition which thereby allows a predominance of excitatory activity, causing tetany and convulsions.

Receptor binding

Until recently most biochemical studies of neurotransmitters in the central nervous system have focussed on presynaptic events. Elegant techniques have been applied to studies of the synthesis of the biogenic amine transmitters, to measurements of their turnover rate, their depolarization-induced release from brain tissue and to their synaptic inactivation by reuptake into the nerve endings which have released them. Many of these approaches have proved valuable in studying amino acid putative neurotransmitters including GABA, glutamic and aspartic acids and glycine. However, little is known of the biochemical properties of the postsynaptic receptors for central nervous transmitters. In the electric organ of the electric eel *Torpedo marmorata* and at vertebrate neuromuscular junctions, a variety of ligands have been employed to demonstrate binding to the nicotinic cholinergic receptor (O'Brien, Eldefrawi & Eldefrawi, 1972, Hall, 1972; Potter & Molinoff, 1972; DeRobertis & Schacht, 1974), and in mammalian brain, it is now possible to identify biochemically muscarinic cholinergic receptors (Soudijn, van Wijngaarden & Ariens, 1973; Burgen, Hiley & Young, 1974; Yamamura, Kuhar, Greenberg & Snyder, 1974; Yamamura & Snyder, 1974).

Using radioactive strychnine, we have successfully measured glycine receptors in the mammalian central nervous system (Young & Snyder, 1973, Snyder, Young, Bennett & Mulder, 1973; Young & Snyder, 1974a,b). Strychnine was chosen as a ligand rather than glycine to avoid possible binding of glycine to presynaptic membrane fragments associated with the glycine uptake system and because neurophysiological studies had suggested that strychnine had a much greater affinity for the glycine receptor than glycine itself. Using a rapid centrifugation technique and tritiated strychnine of high specific activity, we can demonstrate strychnine binding to synaptic membrane preparations of spinal cord and brain stem of the rat and monkey (Young & Snyder, 1973; Snyder *et al.*, 1973; Young & Snyder, 1974a,b). Strychnine binding is most enriched in the synaptic membrane fraction, which contains both pre- and postsynaptic membrane fragments, supporting the notion that strychnine binding is associated with a synaptic receptor site.

In addition, bound [^{3}H]-strychnine is displaced by glycine. Half maximal displacement occurs at about 25 μM glycine with maximal displacement at 1.0 mM glycine. As a measure of strychnine binding to the glycine receptor we define 'specific [^{3}H]-strychnine binding' as the amount of bound [^{3}H]-strychnine which can be displaced by 1 mM glycine. Binding not displaceable by glycine, we define as 'nonspecific strychnine binding'. The ratio of specific to nonspecific binding is about 5-6 at 1-4 nM [^{3}H]-strychnine concentration. The specific binding of [^{3}H]-strychnine is a saturable process with half maximal binding at about 2.7 nM, four orders of magnitude lower than the comparable value for glycine. Nonspecific binding is not saturable and increases linearly with increasing amounts of strychnine. Double-reciprocal and Scatchard analyses indicate that strychnine binds to a single population of receptor sites. The number of strychnine binding sites in the rat spinal cord is about 39 pmol/gram. Interestingly, this is about the same as the number of muscarinic cholinergic receptors in rat brain (Soudijn *et al.*, 1973; Burgen *et al.*, 1974; Yamamura, *et al.*, 1974, Yamamura & Snyder, 1974).

The fact that strychnine binding saturates at low concentrations and can be displaced by glycine is not sufficient evidence to ensure that binding occurs selectively to postsynaptic glycine receptors. As another criterion for specificity, we examined the regional distribution of strychnine binding (Table 1) (Young & Snyder, 1973; Snyder *et al*, 1973; Young & Snyder, 1974a). As already mentioned, endogenous levels of glycine are highest in the spinal cord and brainstem, somewhat lower in the midbrain and diencephalon and lowest in the cerebellum and cerebral hemispheres. Glycine's ability to mimic natural inhibitory synaptic actions is greatest in the brainstem and spinal cord and less in higher centres (Krnjević *et al.*, 1966; Biscoe & Curtis, 1967; Kelly & Krnjević, 1969; Tebēcis & DiMaria, 1972). Thus, one might expect glycine receptors to be most abundant in the spinal cord and brainstem and less in higher centres such as the cerebral cortex. Glycine receptor binding is indeed greatest in the spinal cord and almost as high in the medulla oblongata-pons (Table 1). Receptor binding in the midbrain is only about half that in the medulla oblongata-pons and thalamus, and negligible binding can be demonstrated in the cerebellum, hippocampus, corpus striatum and cerebral cortex. This distribution closely parallels that of endogenous glycine and high affinity glycine uptake systems. Within the spinal cord, endogenous glycine is highest in the cervical and lumbar regions, areas which contain a large proportion of inhibitory interneurones for the regulation of the innervation to the upper and lower extremities. Accordingly, in the monkey's spinal cord specific [^{3}H]-strychnine binding is highest in the cervical and lumbar grey matter and somewhat less in the thoracic grey matter. Receptor binding is undetectable in the white matter of the spinal cord, where few if any synapses occur.

To confirm further the specificity of [^{3}H]-

Table 1 Regional distribution of glycine and strychnine neurophysiological effects, endogenous glycine, glycine high affinity uptake systems and specific [^{3}H]-strychnine binding

	Strychnine-sensitive inhibition[1]	*Glycine hyperpolarization of neurones*[2]	*Endogenous glycine levels*[3] *(µmol/g)*	*High affinity glycine uptake*[4]	*Specific strychnine binding*[5] *(ct min^{-1} mg^{-1} protein)*	
					Rat	*Monkey*
Lumbar spinal cord	+++	+++	4-5	+		1042
Thoracic spinal cord	++	++	3-4	+	2315	775
Cervical spinal cord	+++	+++	4-5	+		886
Medulla oblongata-pons	++	++	3-4	+	1712	—
Midbrain	(?)	(?)	1-2	—	863	—
Thalamus and hypothalamus	(?)	(?)	1	—	500	—
Cerebellum	0	0	1	—	80	—
Cerebral cortex	(±)	(±)	1	—	80	—

1. Data from Curtis *et al.*, 1971. 2. Data from Werman *et al.*, 1968. 3. Data from Aprison *et al.*, 1969a. 4. Data from Bennett *et al.*, 1973. 5. Data from Young & Snyder, 1973.

Table 2 Neurophysiological actions of amino acids and their effects on specific [^{3}H]-strychnine binding

Compound	*% Displacement of bound [^{3}H]-strychnine*** 10^{-5} M	10^{-4} M	10^{-3} M	*Neurophysiological activity of amino acids**	*Strychnine antagonized depression of spinal dorsal horn interneurones*
α-Amino acids					
Glycine	50	84	100	— — — —	yes
L-α-Alanine	5	40	64	— —	yes
L-Serine	6	17	56	—	yes
L-Cystathionine	—	—	—	(—)	yes
Aminomethane sulphonic acid	0	7	38	0	yes
Proline	0	12	61	not tested	not tested
Glutamic acid	0	0	0	++++	no
Aspartic acid	0	0	0	++++	no
Tryptophan	—	—	47	not tested	not tested
Phenylalanine	—	—	31	not tested	not tested
β-Amino acids					
β-Alanine	50	85	100	— — —	yes
DL-β-Aminoiso butyric acid	26	47	100	— —	yes
Taurine	14	68	100	— —	yes
γ-Amino acids					
γ-Aminobutyric acid	0	9	24	— — —	no
γ-Amino-β-hydroxybutyric acid	—	—	21	— —	no
Higher -amino acids					
Δ-Aminovaleric acid	—	—	28	— —	no
ϵ-Aminocaproic acid	—	—	20	—	no

Amino acids having no effect at 10^{-3} M: isoleucine, cysteine, valine, histidine, methionine, threonine, lysine, arginine, and leucine.

* Curtis *et al.*, 1961; 1968: (—) Indicates relative depressant activity relative to glycine. (+) Indicates relative excitatory activity.

** See Young & Snyder, 1973, 1974a.

strychnine binding we compared the ability of a variety of amino acids to displace bound [^{3}H]-strychnine with their ability to mimic the strychnine-sensitive neurophysiological actions of glycine (Table 2). β-Alanine and glycine are the most potent displacers of [^{3}H]-strychnine and are also the most potent amino acids neurophysiologically. Taurine, DL-β-aminoisobutyric acid and L-α-alanine are second most potent both neurophysiologically and as inhibitors of [^{3}H]-strychnine binding. Serine is somewhat less effective as a displacer of bound strychnine and has a limited ability to mimic glycine neurophysiologically. Most of the other amino acids examined have negligible ability to block strychnine binding and are unable to reproduce the synaptic actions of glycine in neurophysiological studies.

The close parallel between the regional distribution of specific strychnine binding, endogenous glycine levels and glycine's neurophysiological actions, as well as the impressive correlation between the neurophysiological actions of glycine-like amino acids and their ability to inhibit [^{3}H]-strychnine binding, indicate that specific [^{3}H]-strychnine binding involves synaptic recpetor sites for glycine.

Apparent interactions of strychnine with the ionic conductance mechanism of the glycine receptor

It is thought that neurotransmitters interact with a specific recognition site on the postsynaptic receptor thereby triggering alterations in a closely associated ion channel or 'ionic gate'. This causes a change in the permeability of the membrane to a particular ion or ions, and an alteration in membrane conductance which results in either neuronal depolarization or hyperpolarization. For excitatory neurotransmitters, such as acetylcholine at the neuromuscular junction, the ion mediating the depolarizing effects of acetylcholine seems to be predominantly sodium (Fatt, 1954; Katz, 1966). For inhibitory neurotransmitters such as GABA and glycine the transmitter appears to bring about an increase in conductance to chloride and possibly potassium which effects a hyperpolarization of the membrane (Ito, Kostyk & Oshima, 1962; Eccles, 1964). A major aim of biochemical studies of neurotransmitter receptors involves the identification of the receptor recognition site and ionic conductance mechanisms in order to ascertain on a molecular basis how neurotransmitter recognition at one site triggers an alteration in the ionic permeability of the neuronal membrane. A variety of ligands can be used to label the recognition site of neurotransmitter receptors (O'Brien *et al.*, 1972; Hall, 1972; Potter & Molinoff, 1972; Soudijn *et al.*, 1973; Burgen *et al.*, 1974; DeRobertis & Schacht, 1974; Yamamura *et al.*, 1974; Yamamura & Snyder, 1974) and recently, the neurotoxin, histrionicotoxin, has been shown to interact with the ionic conductance modulator of the nicotinic acetylcholine receptor at neuromuscular junctions (Albuquerque, Barnard, Chiu, Lapa, Dolly, Jansson, Daly & Witkop, 1973). However, it is not yet possible to study biochemically the binding of histrionicotoxin to these sites.

We first suspected that glycine and strychnine might bind to different sites on the glycine receptor when we examined the influence of a variety of protein modifying reagents on [^{3}H]-strychnine binding and its displacement by glycine (Table 3). Diazonium tetrazole and acetic anhydride have very little effect on the total amount of [^{3}H]-strychnine bound to synaptic membranes. However, both of these reagents markedly interfere with the ability of glycine to displace [^{3}H]-strychnine binding. By contrast, tetranitromethane and dinitrofluorobenzene inhibit the total binding of [^{3}H]-strychnine but glycine and strychnine still displace the remaining bound [^{3}H]-strychnine to similar extents. The differential influence of diazonium tetrazole and acetic anhydride on strychnine and glycine interactions with the receptor suggest that glycine might displace strychnine by acting at site other than the strychnine binding site.

If the effect of diazonium tetrazole is to modify residues that are necessary for the interaction of the glycine site with a distinct strychnine binding site, it might be possible to 'protect' these residues by pretreating the membranes with diazonium tetrazole in the presence of glycine. Indeed, glycine can completely protect the membranes from the effects of diazonium tetrazole (Table 3). By contrast strychnine, even at 1 mM concentration, affords no protection of the membranes from diazonium tetrazole treatment. In fact, it seems to enhance slightly the ability of the reagent to inhibit glycine displacement.

These data suggest that glycine and strychnine bind to two separate but mutually interacting sites. If this is so, analysis of their binding interactions at various concentrations might be anticipated to demonstrate cooperative interactions. Hill plots for the displacement of [^{3}H]-strychnine or glycine are linear with an *n* for displacement with strychnine of 1.0, while the *n* for displacement with glycine is 1.7 (Young & Snyder, 1974b). This suggests that glycine interacts with strychnine in a cooperative fashion.

If the preferential influence of diazonium tetrazole on glycine's displacement of strychnine binding derives from an interference with interactions between glycine and strychnine binding sites, one might expect diazonium tetrazole pretreatment to alter the cooperative effects of glycine on strychnine binding. Acordingly, Hill plots were performed for the displacement of [^{3}H]-strychnine binding by glycine using synaptic membranes pretreated with low concentrations of diazonium tetrazole which reduce specific strychnine binding less than 30%. Interestingly, diazonium tetrazole pretreatment of the synaptic membranes reduces the Hill coefficient for strychnine displacement by glycine from a value of 1.7 in control membranes to values of 1.2 and 1.1 after pretreatment with 0.25 mM and 0.75 mM diazonium tetrazole respectively. Acetic anhydride pretreatment similarly reduces the Hill coefficient for displacement of strychnine binding by glycine from 1.7 to 1.2 (Young & Snyder, 1974a).

Finally, we measured the dissociation rate of the [^{3}H]-strychnine-receptor complex before and after pretreatment with two concentrations of diazonium tetrazole. The dissociation of [^{3}H]-strychnine is unaffected by diazonium tetrazole pretreatment when measured after the addition of unlabelled strychnine at time zero. However, the dissociation rate when displacement is measured after the addition of glycine is considerably retarded in the diazonium tetrazole-pretreated membranes. The half-life for dissociation in control membranes after glycine addition is 45 s, while the half-lives using membrane preparations pretreated with 0.25 mM and 0.75 mM diazonium tetrazole are 56 s and 66 s, respectively (Young & Snyder, 1974a). These results indicate further that glycine and strychnine binding can be differentiated and that diazonium tetrazole may interfere with interactions between their binding sites.

Thus strychnine and glycine apparently bind to different portions of the receptor. Glycine presumably interacts with the glycine recognition site. To what structure does strychnine bind? Our first clue came in evaluating the effects of a variety of ions on [^{3}H]-strychnine binding. We found that in the absence of sodium, physiological concentrations of chloride markedly reduced specific [^{3}H]-strychnine binding. Since glycine-induced hyperpolarization of the neuronal membrane results primarily from a change in conductance of the membrane to chloride, we wondered whether strychnine might bind to the chloride ionic conductance modulator of the glycine receptor. Accordingly, we studied the influences of a variety of anions on strychnine binding. Numerous ions have been evaluated for their ability to substitute for chloride neurophysiologically during transmitter-induced hyperpolarization of the neuronal membrane. Thus when certain anions are injected iontophoretically into spinal motor neurones, the cell is hyperpolarized beyond the reversal potential for the transmitter-induced conductance change in the neuronal membrane. When inhibitory inputs to the cell are then stimulated, a depolarization results, presumably because the ion can traverse the chloride ionic gate which is 'opened' by neurotransmitter-receptor interactions. Ions which cannot traverse these channels fail to cause a reversal of the inhibitory postsynaptic potential. Neurophysiologically, ions behave in an all or none fashion; either they are fully effective in reversing the inhibitory postsynaptic potential or they are

Table 3 Effects of protein modifying reagents on specific [^{3}H]-strychnine binding

Reagent		*Total [^{3}H]-strychnine binding (% control)*	*Strychnine displaceable [^{3}H]-strychnine binding (% control)*	*Glycine displaceable [^{3}H]-strychnine binding (% control)*
None		100	100	100
2,4-Dinitrofluorobenzene	4.0 mM	37	28	26
Tetranitromethane	5.0 mM	52	42	40
N-ethylmaleimide	4.0 mM	93	92	91
Acetic anhydride	10 mM	100	98	69
	25 mM	98	84	29
	50 mM	70	51	10
Diazonium tetrazole	0.25 mM	100	98	90
	0.75 mM	95	93	70
	1.00 mM	90	85	33
	1.25 mM	90	60	18
Diazonium tetrazole,	1.0 mM			
+ glycine	1.0 mM	96	94	81
+ strychnine	1.0 mM	80	64	0

Data are modified from Young & Snyder, 1974a.

ineffective. The neurophysiological action of the anions appears to correlate with their hydration radii such that neurophysiologically active anions have hydration sizes similar to or smaller than chloride, while anions with larger hydration radii are inactive. The only striking exception to this sequence is formate, whose hydration radius is 40% larger than that of chloride, but which is effective in inverting inhibitory postsynaptic potentials, while several other anions with hydration radii larger than chloride but smaller than formate are neurophysiologically inactive (Ito *et al.*, 1962; Eccles, 1964).

We evaluated a series of 14 anions whose neurophysiological actions on inhibitory postsynaptic potentials in spinal motorneurones are known (Young & Snyder, 1974b) (Table 4). Their ability to inhibit [^{3}H]-strychnine binding to spinal cord synaptic membranes correlates closely with their neurophysiological capacity to invert inhibitory postsynaptic potentials in the mammalian spinal cord. Seven neurophysiologically active ions are able to inhibit specific [^{3}H]-strychnine binding, while seven neurophysiologically ineffective ions fail to alter strychnine binding. While effects on strychnine binding tend to correlate with the hydration radii of the ions, the correlation is better with the neurophysiological properties, since formate, whose hydration radius is 40% greater than chloride, is active neurophysiologically and in inhibiting strychnine binding.

The close correlation between the effects of anions on strychnine binding and on inhibitory postsynaptic potentials in mammalian spinal cord suggests that strychnine binding is closely associated with the chloride ionic conductance mechanism of the glycine receptor. Whether or not strychnine binds directly to the chloride 'gate' is unclear. Hill plots of the displacement of [^{3}H]-strychnine by chloride, iodide and bromide give coefficients of 2.3-2.7, suggesting that chloride interacts with strychnine binding in a cooperative fashion, which might mean that the two compounds bind to different sites. It is also possible, however, that strychnine binds to a site such as the exterior of the chloride channel and, to displace it, several chloride molecules must occupy the interior of the channel. In any case, we know of no feature of the glycine receptor other than the ionic gating mechanism which can account for the close relationship between anion effects on strychnine binding and on inhibitory postsynaptic potentials.

The fact that strychnine binding might be associated with the ionic conductance mechanism for chloride while glycine interacts with the glycine recognition site provides us with a tool to examine the interactions between these two portions of the glycine receptor. Recently, we have developed techniques for measuring the direct binding of glycine to the receptor and have also succeeded in solubilizing the glycine receptor. It may now be possible to separate physically the glycine and strychnine binding moieties of the receptor, to characterize them individually and to study their interactions.

Table 4 Effects of various anions on specific [^{3}H]-strychnine binding and reversal of the inhibitory postsynaptic potential (ipsp)

*Anion**	*ED$_{50}$ for inhibition of specific [^{3}H]-strychnine binding*	*Hydration radii (22)*	*Reversal of the ipsp (22)*
Formate	160	1.35***	+
Bromide	235	0.94	+
Chloride	260	0.95	+
Iodide	300	0.96	+
Nitrate	335	1.03	+
Perchlorate	440	1.09	+
Thiocyanate	620	1.12	+
Fluoride	— —**	1.33	—
Bicarbonate	— —**	1.65	—
Acetate	— —**	1.80	—
Sulphate	— —**	1.85	—
Sulphite	— —**	2.05	—
Phosphate, monobasic	— —**	2.05	—
Phosphate, dibasic	— —**	2.56	—

* Ammonium salts of all anions were used (Data are from Young & Snyder, 1974b).

** No inhibition detectable at 1.0 M concentrations.

*** Hydration radii expressed relative to potassium's which is given the value of unity (Eccles, 1964).

The notion that glycine interacts in a cooperative fashion with strychnine binding and that the cooperativity may be between the recognition site and the ionic gate of the receptor may have implications for elucidating synaptic features of other neurotransmitters. In several systems, the neurophysiological synaptic actions of glycine, acetylcholine and GABA display cooperative features (Changeux & Podleski, 1968; Takeuchi & Takeuchi, 1969; Davidoff, Aprison & Werman, 1969, Brookes & Werman, 1970; 1973). At the neuromuscular junction of the crayfish, Takeuchi & Takeuchi (1969) have examined interactions between GABA and picrotoxin, a GABA receptor blocker. GABA hyperpolarizes the crustacean neuromuscular junction in a cooperative fashion with Hill coefficients of 1.9, whereas the Hill coefficient for picrotoxin blockade of GABA effects is 1.0 and picrotoxin appears to block GABA effects non-competitively. This is very reminiscent of our data differentiating the glycine and strychnine binding sites of the glycine receptor. In further analogy, Takeuchi & Takeuchi (1969) observe that increasing concentrations of chloride reduce the ability of picrotoxin to block GABA effects. Half maximal inhibition occurs at about 200 mM chloride concentrations, similar to the half maximal concentration needed for inhibiting strychnine binding to the glycine receptor. Conceivably, at this GABA receptor the cooperative interactions take place between the GABA recognition sites and the chloride ionic gate, where picrotoxin may act. Such detailed neurophysiological studies have not yet been performed for glycine.

Cooperativity of neurotransmitter actions may serve a valuable physiological function. Because of the sigmoid character of the dose-response curve, the membrane potential of the postsynaptic neurone would be protected from small amounts of transmitter which might leak into the synaptic cleft but fail to trigger a change in membrane response because of the cooperativity of the dose-response curve. Similarly, the mechanism responsible for terminating the synaptic actions of a transmitter need not be 100% efficient, since it would only have to reduce the concentration of transmitter in the synaptic cleft below a threshold level and would not be obliged to 'sweep' the cleft free of every last transmitter molecule.

Further, the sigmoidicity of the transmitter-receptor dose-response curve may have implications for the mechanism of action of certain pharmacological agonists. Thus doses of drug that do not in themselves activate the receptor complex may have marked effects on function by increasing the ambient level of active agent in the synaptic cleft to near threshold levels. Then, when a natural pathway is stimulated, a smaller amount of transmitter release is necessary to reach threshold concentrations and activate the postsynaptic receptor. By such mechanisms, an agonist drug, in proper doses, might have no intrinsic effect on neuronal function but would instead serve to enhance the effectiveness of transmission in certain endogenous transmitter pathways.

Mechanism of benzodiazepine action

Many psychotropic drugs are known to act via the biogenic amine neurotransmitters. Except for convulsants such as picrotoxin and strychnine, no drugs have hitherto been shown to act as agonists or antagonists of amino acid neurotransmitters. The availability of a simple and specific assay for the glycine receptor prompted us to screen a large number of psychotropic drugs for the ability to inhibit [^{3}H]-strychnine binding (Young, Zukin & Snyder, 1974). Many drugs examined had negligible effects on strychnine binding (Table 5). The benzodiazepines, such as diazepam (Valium), are as potent in inhibiting [^{3}H]-strychnine binding as glycine itself (Young *et al.*, 1974). Diazepam inhibits strychnine binding 50% at a concentration of about 26 μM. However, the fact that a drug exerts an effect upon a particular biochemical parameter by no means establishes that this is the mechanism of the drug's therapeutic action. To determine whether benzodiazepine's interactions with the glycine receptor are related to the clinical effects of the drug, we examined a series of 21 benzodiazepines whose pharmacologic activities have been extensively evaluated in animal tests and in humans (Zbinden & Randall, 1967; Morselli, Cassano, Piacidi, Muscettola & Rizzo, 1973) (Table 5). There is a 50-fold variation in the potency of these drugs in inhibiting [^{3}H]-strychnine binding with values of half maximal inhibition ranging from 19 μM to more than 1000 μM. Diazepam (Valium) and chlordiazepoxide (Librium), the two most frequently prescribed drugs in the United States have ED_{50} values of 26 μM and 200 μM respectively.

To determine if the interactions of benzodiazepines with the glycine receptor relate to their clinical activities, we compared the clinical potency of the drugs in pharmacological tests that predict clinical activity with their potency in displacing [^{3}H]-strychnine binding (Table 5). The ability of the drugs to displace bound [^{3}H]-strychnine correlates very closely with their potencies in a 'human bioassay' based on the minimal dose at which 50% of the subjects experience subjective effects. Similar close correlations occur between strychnine displacement and the potencies of the drugs in several behavioural tests in animals. These tests, such as the mouse antifighting, monkey taming, mouse and cat muscle relaxation, antipentylenetetrazole seizure and rat continuance avoidance tests are effective predictors of the drug potencies in humans. Considerably lower correlations are obtained with discrete trial conditioning, with the effects of the drugs on escape failure from electric shock, and convulsions elicited by minimal or maximal electroshock. However, these tests are also less effective predictors of benzodiazepine action in humans (Zbinden & Randall, 1967; Morselli *et al.*, 1973).

The close correlation between the pharmacological activity of the benzodiazepines and their displacement of strychnine binding strongly suggests that the drugs exert their pharmacological activities by interacting with the glycine receptor. However, it is still conceivable that the relative pharmacological potencies of the drugs *in vivo* relate simply to their ability to reach brain receptors because of factors such as lipid

Table 5 Benzodiazepines: correlation of $[^3H]$-strychnine binding displacement with behavioural effects

Behavioural test	*No. of drugs tested*	*Statistical significance* (P)	*Spearman correlation coefficient*
Human bioassay	20	<0.001	0.74
Fighting mouse test	21	<0.001	0.71
Antipentylene tetrazole test, mice	20	<0.004	0.63
Continuous avoidance, shock rate increase, rat	20	<0.004	0.63
Cat muscle relaxation	19	<0.005	0.67
Monkey taming	19	<0.005	0.65
Mouse muscle relaxation	20	<0.005	0.61
Antimaximal electroshock, mice	19	<0.05	0.46
Discrete trials 'trace' avoidance, noise response failure, rat	14	<0.10	0.51
Antiminimal electroshock, mice	19	<0.20	0.33
Continuous avoidance, escape failure, rat	19	<0.45	0.20

Central muscle relaxants without effect on $[^3H]$-strychnine binding at 10^{-3} M

Carisoprodol
Chloroxazone
Meprobamate
Methocarbamol
Mephenesin
Methaqualone
Tybamate

Drugs without effect on $[^3H]$-strychnine binding at 10^{-4} M

Tetracycline
Aminosalicylic acid
Nicotinamide
Pyridoxal HCl
Dilantin
α-Naphthylthiourea
Urea
Acetazolamide
Ethoxyzolamide
Methyl-DOPA
Ergocristine
Methysergide
Hippuric acid
Melatonin
Tolbutamide
Alloxan
Ketamine
Lithium carbonate
Ethanol
Reserpine
Benactyzine
Haloperidol
Hydroxyzine
Diethyl carbamazine
Lidocaine HCl
Dichlorphenamide
Chlorphenesin
Ethosuximide
Phenacemide
Carbamazepine
Acetophenetidine

Data are adapted from Young *et al.*, 1974.

solubility. Such effects might also determine the access of the drugs to strychnine binding sites in our synaptic membrane preparations. If this is the case, the same factors should govern interactions of benzodiazepines with other central nervous system receptors *in vitro*. To examine this possibility, we measured the ability of 10 of the benzodiazepines, including the most and least potent, to interact with opiate and muscarinic cholinergic brain receptors (Soudijn *et al.*, 1973; Pert & Snyder, 1973; Burgen *et al.*, 1974; Yamamura *et al.*, 1974; Yamamura & Snyder, 1974, Pasternak & Snyder, 1974). Displacement of [^{3}H]-dihydromorphine binding to opiate receptor sites or displacement of binding of [^{3}H]-quinuclidinyl benzilate to muscarinic cholinergic receptors in membrane preparations of rat brain requires concentrations of benzodiazepines 10-50 times higher than those needed for displacement of bound [^{3}H]-strychnine. Further, the inhibition of opiate and muscarinic receptor binding by benzodiazepines fails to correlate with any of the pharmacological tests. Thus, it is highly unlikely that the correlation of pharmacological activity of benzodiazepines with their ability to displace [^{3}H]-strychnine binding can be explained simply by nonspecific membrane interactions.

Drugs other than the benzodiazepines, most notably propanediols such as meprobamate (Miltown, Equanil), also exert muscle relaxant effects. These agents fail to displace [^{3}H]-strychnine binding in concentrations as high as 10 mM. However, the bearing of these data on the mechanism of action of these drugs must be viewed with caution, since these drugs are pharmacologically quite weak *in vivo* compared to the benzodiazepines. For example, in clinical usage meprobamate is less than 1% as potent as diazepam.

The major clinical actions of the benzodiazepine drugs are relief of anxiety and muscle relaxation. Since the anxiety relieving properties of these drugs are closely correlated with their muscle relaxing actions, the latter might be responsible for the amelioration of anxiety. Alternatively, antianxiety and muscle relaxant effects might be exerted at different parts of the central nervous system but utilize a common mechanism. Glycine neurones and receptors exist in both the spinal cord and brainstem, and in diencephalic structures. The concentrations of benzodiazepines in the blood and central nervous system at pharmacologically active doses (Zbinden & Randall, 1967, Morselli *et al.*, 1973) is similar to the concentration required to displace [^{3}H]-strychnine binding. Because of the impressive correlation of the pharmacological activity of the benzodiazepines and their interactions with central nervous glycine receptors, we propose that these drugs may produce their antianxiety, anticonvulsant and muscle relaxant effects by mimicking the actions of glycine at its receptor sites in the central nervous system. Conceivably, muscle relaxant actions involve an enhancement of glycine-mediated inhibition in the spinal cord, while antianxiety effects result from enhanced synaptic inhibition in the brain stem or higher centers. Influences of benzodiazepines on limbic system activity (Zbinden & Randall, 1967) might involve glycine receptors in limbic structures (Young & Snyder, 1973; Snyder *et al.*, 1973; Young *et al.*, 1974) or might arise secondarily from effects on brainstem receptors. The effects of benzodiazepines on seizure activity, both cortical and subcortically induced, may result from potentiating inhibitory pathways from the brainstem reticular activiting systems.

Concluding remarks

The ability to identify biochemically the synaptic receptor for glycine in the central nervous system has valuable implications for understanding synaptic mechanisms and for elucidating the mechanism of action of certain drugs. The notion that cooperative interactions exist between the glycine recognition site and its ionic conductance mechanism is reminiscent of neurophysiological results obtained for interactions between GABA and picrotoxin. Perhaps such synaptic mechanisms may exist for a variety of neurotransmitters. Similarly the apparent ability of the benzodiazepine drugs to exert their clinical effects by mimicking glycine at its receptors suggests that studying the influence of psychotropic drugs on synaptic actions of other amino acid neurotransmitters will be heuristic.

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E. coli ENDOTOXIN SHOCK IN THE CAT; TREATMENT WITH INDOMETHACIN

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1 An earlier study had demonstrated that indomethacin, administered before *E. coli* endotoxin, abolished the initial pulmonary vasoconstriction and delayed the onset of the secondary shock phase that results from the intravenous injection of this agent in cats. The object of the present study was to determine whether indomethacin modified the shock phase when administered after endotoxin.

2 All the cats (whether or not they received indomethacin, 10 mg/kg) exhibited the characteristic features of the delayed shock phase that result from the administration of endotoxin (2 mg/kg). These included systemic hypotension, hypoglycaemia, reductions in arterial pH, cardiac output and systolic ejection time and an increase in arterial lactate. Five out of the ten animals given indomethacin survived 4 h compared with four out of twelve in the control (endotoxin alone) group.

3 These results do not support the suggestion that antipyretic-analgesic drugs like indomethacin may be of benefit when given during bacteraemic or septic shock. They do support the suggestion that the acute pulmonary changes (hypertension and decreased compliance) that occur in this species within a few minutes of endotoxin administration ultimately contribute to the severity of the shock phase.

Introduction

The results of a previous study (Parratt & Sturgess, 1974) demonstrated that pretreatment with indomethacin had two effects on the response of cats to *E. coli* endotoxin. Firstly, it prevented the acute pulmonary hypertension and oedema that occur within 1-2 min of endotoxin administration. Secondly, it delayed the systemic hypotension (a characteristic of the shock phase) and significantly increased survival time (e.g. 6 out of 15 cats were alive at 6 h compared with only 1 out of 25 in the control endotoxin series). More relevant to any possible clinical application of these experimental results is the question whether antipyretic-analgesic drugs modify the course of endotoxin-induced shock once it has started. The purpose of the present study was to attempt to answer this question.

Methods

Twenty-two cats of either sex were anaesthetized with sodium pentobarbitone (30 mg/kg by intraperitoneal injection) and ventilated with room air using a Palmer positive-pressure pump. The stroke volume of the pump was adjusted after thoracotomy to give an adequate degree of oxygenation (mean arterial $P\text{O}_2$ 80 ± 4 mmHg); to achieve this a degree of hyperventilation was necessary (Parratt & Sturgess, 1974). No attempt was made to alter stroke volume during the course of the experiments. Rectal and mid-oesophageal temperatures were recorded with direct recording thermocouples (Ellab, Copenhagen). Systemic arterial pressure, pulmonary artery pressure and the electrocardiogram were measured as previously described (Parratt, 1973) and recorded on an ink-jet writing recorder (Elema-Schönander Mingograph 81). Mean pulmonary artery pressure was obtained by electronic integration, systolic ejection time was measured from the beginning of the upstroke of the central aortic pressure pulse to the trough of the incisural notch and cardiac output was measured by thermodilution. Arterial blood samples were analysed for O_2 and CO_2 tensions and pH with appropriately calibrated electrode systems (Radiometer, Copenhagen). Arterial lactate was measured by the Hohorst enzymatic method and plasma glucose by means of a Beckman analyser.

All the cats received, by slow intravenous injection, 2 mg/kg of the same batch of *E. coli* endotoxin (Difco, 055:B5) suspended in 0.9% w/v sodium chloride solution. Thirty minutes later ten of the cats received indomethacin (10 mg/kg, dissolved in a phosphate buffer pH 8) injected slowly into a femoral vein. The various cardiovascular and metabolic parameters were measured each hour after the administration of endotoxin.

Results

The administration of endotoxin produced a marked elevation of pulmonary arterial pressure, with the maximum effect 3-4 min after the end of the injection. The degree of pulmonary hypertension was similar in the two groups i.e. those that were to serve as controls (no further treatment) and those that were to receive indomethacin. The values were (in the control group) 19 ± 1 mmHg (systolic); 10 ± 1 mmHg (diastolic) and 13 ± 1 mmHg (mean) immediately before endotoxin and 40 ± 5 mmHg; 23 ± 3 mmHg and 30 ± 4 mmHg respectively 3 min after endotoxin administration. In the group that were later to receive indomethacin the corresponding values were 23 ± 1 mmHg (systolic); 11 ± 1 mmHg (diastolic) and 15 ± 1 mmHg (mean) before endotoxin and 43 ± 4, 28 ± 3 and 32 ± 3 mmHg respectively 3 min after endotoxin. All these changes were significant ($P < 0.001$). There were also similar effects in the two groups with regards to systemic hypotension, bradycardia and the occurrence of ventricular arrhythmias; these initial effects of endotoxin have been described in detail in earlier studies (Parratt, 1973; Parratt & Sturgess, 1974).

When injected slowly 30 min after the endotoxin, indomethacin had no significant effect on systemic blood pressure (97 ± 8 mmHg systolic, and 58 ± 5 mmHg diastolic, immediately before, and 101 ± 12 mmHg and 57 ± 9 mmHg 5 min after, the administration of indomethacin), or on heart rate (216 ± 10 and 208 ± 11 beats/min) or mean pulmonary pressure (17 ± 2 mmHg and 16 ± 2 mmHg). There was no evidence of the hypertensive response which has been described following indomethacin administration in the dog subjected to haemorrhagic or endotoxin shock (Collier, Herman & Vane, 1973).

The cardiovascular effects of *E coli* endotoxin are summarized in Table 1. There was a developing systemic hypotension, a reduction in systolic ejection time, in cardiac output and in stroke volume (1.6 ± 0.2 ml/beat before endotoxin and 1.2 ± 0.4 ml/beat at 3 hours). There was also a substantial metabolic acidosis, with a three to four-fold increase in arterial lactate (Table 3) and a reduction in arterial $P\text{O}_2$, probably as a result of the pulmonary oedema and increased shunting that occurs after endotoxin administration in this species (Parratt, 1973). Although there was a considerable individual variation, the plasma glucose tended to fall during the shock phase (Table 3). This hypoglycaemic response has also been observed in the dog and in man (Berk, Hagen, Beyer & Gerber, 1970; Griffiths, Groves & Leung, 1973). Of the 12 cats administered endotoxin alone four were alive at 4 h and one of these survived to 6 h, these results are similar to those obtained in the earlier series (Parratt & Sturgess, 1974).

The results of administering indomethacin after the endotoxin shock phase had started are summarized in Tables 2 and 3. There was probably

Table 1 Haemodynamic effects of *E. coli* endotoxin (2 mg/kg i.v.) in anaesthetized cats

	Control (pre-endotoxin)	*Post-endotoxin*				
		1 h	*2 h*	*3 h*	*4 h*	*5 h*
Carotid artery pressure						
(systolic; mmHg)	105 ± 6	104 ± 10	87 ± 12	90 ± 14	89 ± 23	102
(diastolic; mmHg)	76 ± 6	61 ± 10	48 ± 8[*]	54 ± 11	51 ± 14	40
Pulmonary artery pressure (mean; mmHg)	13 ± 1	16 ± 2	15 ± 2	16 ± 1	16 ± 1	16
Heart rate (beats/min)	191 ± 12	239 ± 15	218 ± 14	204 ± 15	200 ± 25	180
Systolic ejection time (ms)	130 ± 9	100 ± 10	101 ± 7[*]	94 ± 10[*]	107 ± 14	125
Cardiac output ($\text{ml min}^{-1}\ \text{kg}^{-1}$)	132 ± 18	126 ± 13	89 ± 25	103 ± 37	–	120
Peripheral vascular resistance (arbitrary units)	2.7 ± 0.3	2.6 ± 0.5	3.4 ± 0.9	3.4 ± 2.0	–	2.7
Number of survivors	12	10	10	7	4	1[+]

[+] This animal survived 6 hours
[*] $P < 0.02$.

Table 2 Haemodynamic effects of *E. coli* endotoxin (2 mg/kg i.v.) and indomethacin (10 mg/kg) when administered 30 min after the endotoxin

	Control	*Post-endotoxin*				
	(pre-endotoxin)	*1 h*	*2 h*	*3 h*	*4 h*	*5 h*
Carotid artery pressure						
(systolic; mmHg)	115 ± 8	104 ± 10	103 ± 13	107 ± 12	100 ± 12	83
(diastolic; mmHg)	86 ± 8	61 ± 7*	66 ± 11	73 ± 10	66 ± 13	56
Pulmonary artery pressure (mean; mmHg)	15 ± 1	16 ± 1	16 ± 1	15 ± 1	16 ± 1	14
Heart rate (beats/min)	215 ± 13	221 ± 11	217 ± 14	235 ± 15	240 ± 14	223
Systolic ejection time (ms)	118 ± 8	102 ± 7	95 ± 7*	86 ± 9*	88 ± 11*	—
Cardiac output ($ml\ min^{-1}\ kg^{-1}$)	101 ± 14	92 ± 13	93 ± 16	79 ± 14	81 ± 12	77
Peripheral vascular resistance (arbitrary units)	3.7 ± 0.5	3.9 ± 0.5	4.2 ± 0.9	4.6 ± 0 8	3.8 ± 0.7	2.1
Number of survivors	10	9	8	6	5	3+

+ One animal survived 6 hours.
* $P < 0.05$.

Table 3 Comparison of arterial oxygen tension, pH, arterial lactate and plasma glucose in cats administered *E. coli* endotoxin. Those in Group B also received indomethacin (10 mg/kg i.v.) 30 min after the endotoxin

	Control		*Post-endotoxin*					
			1 h		*2 h*		*3 h*	
Group	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*
Po_2 (mmHg)	83 ±6	77 ±3	69 ±5*	60 ±5*	84 ±8	66 ±7	89 ±11	72 ±9
Pco_2 (mmHg)	22 ±2	20 ±4	27 ±4	22 ±2	24 ±6	22 ±3	22 ±4	19 ±3
pH (units)	7.500 ±0.059	7.487 ±0.043	7.346 ±0.067	7.257 ±0.062*	7.293 ±0.032*	7.167 ±0.078*	7.232 ±0.082*	7.285 ±0.031**
Lactate (mg/100 ml)	7.6 ±1.1	9.4 ±1.9	23.5 ±3.0**	20.6 ±2.1*	37.6 ±4.0**	32.3 ±4.8**	41.4 ±6.7**	30.8 ±4.0**
Glucose (mg/100 ml)	115 ±11	103 ±11	119 ±29	119 ±17	97 ±20	108 ±33	59 ±21*	95 ±14

Values are mean ± s.e.mean. Group A $n = 12$, group B $n = 10$.
The corresponding values for spontaneously breathing cats are: Po_2 94 ± 3 mmHg; Pco_2 27 ± 2 mmHg; pH 7.335 — 0.020 units; lactate 6.7 ± 0.9 mg/100 ml and glucose 106 ± 6 mg/100 ml.
* $P < 0.01$; ** $P < 0.001$.

a tendency for the systemic blood pressure to be maintained for longer than in the control group (compare the diastolic blood pressure values at 2, 3 and 4 h in Tables 1 and 2) but this was certainly not as marked as when indomethacin was given prophylactically (Parratt & Sturgess, 1974). Cardiac output fell to the same extent as in the cats given endotoxin alone and there was no modification of the endotoxin-induced metabolic acidosis (Table 3) or reduction in arterial Po_2. Five of the ten animals survived 4 h compared with four out of 12 in the control group. Only one animal survived 6 hours.

Discussion

This study was undertaken because of the observation that pretreatment with indomethacin increased survival following *E. coli* endotoxin in the cat (Parratt & Sturgess, 1974), a result similar to that obtained with this, and with other antipyretic-analgesic drugs (sodium salicylate, acetylsalicylic acid, flufenamic acid and sodium α-4-*sec*-butylphenoxypropionate) in the dog (Northover & Subramanian, 1962; Erdős, 1968; Culp, Erdös, Hinshaw & Holmes, 1971). Clearly such studies are not relevant to the question whether such drugs might be used in the treatment of developed septic or bacteraemic shock in man although, because it prevents endotoxin-induced pulmonary changes in cats, the use of aspirin has been suggested as a therapeutic measure to help prevent further deterioration of established pulmonary damage in patients with gram-negative septicaemia (Murthy & Greenway, 1972). We would suggest that because therapeutic success in septic shock is far from satisfactory (the mortality in patients with gram-negative bacteraemia is about 40%; Barnett & Sanford, 1969) any new approach to the problem should be actively investigated. In this respect the present results were disappointing. All the cats exhibited the characteristic features of the shock phase i.e. a reduced arterial oxygen tension, a marked metabolic acidosis and reductions in systolic ejection volume and time and in cardiac output. There was no clear evidence of increased survival. This is in contrast to the effect of pretreatment with indomethacin (Parratt & Sturgess, 1974) and suggests that the acute pulmonary changes (hypertension and decreased compliance) that occur in this species within a few minutes of endotoxin administration ultimately contribute to the severity of the shock phase. If these changes are prevented by the prior administration of indomethacin, survival time is increased despite the severity of the secondary shock phase. These results might also suggest that the beneficial effects of indomethacin pretreatment result from inhibition of prostaglandin biosynthesis or of platelet aggregation rather than to stabilization of lysosomal membranes, since the latter would presumably also occur when indomethacin is administered during the shock phase.

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THERMOREGULATORY CHANGES INDUCED BY CHOLINOMIMETIC SUBSTANCES INTRODUCED INTO THE CEREBRAL VENTRICLES OF SHEEP

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1 Thermoregulatory responses have been recorded from Welsh Mountain sheep exposed to warm, neutral or cold environments while injections of cholinomimetic drugs and/or their antagonists have been given into a lateral cerebral ventricle.

2 Carbachol and physostigmine inhibited panting of animals at high ambient temperature (t_a), caused vasoconstriction and initiated shivering at neutral t_a, and accentuated shivering at low t_a. Rectal temperature (t_{re}) invariably increased. Oxotremorine had apparently identical effects.

3 Nicotine and another ganglionic stimulant, the quaternary methyl derivative of dopamine, had no effects on thermoregulation.

4 Atropine given 10 min before injections of carbachol, physostigmine or oxotremorine completely inhibited their hyperthermic effects, but pretreatment with the ganglion-blocking drug, pempidine, caused no inhibition. The cholinergic synapses that respond to cholinomimetic drugs injected into the lateral cerebral ventricles of sheep are therefore muscarinic and not nicotinic.

5 When atropine was given to sheep exposed to cold, no detectable reduction of shivering occurred and t_{re} decreased only slightly, even with doses of atropine far greater than needed to inhibit shivering induced by physostigmine. This may be because shivering is controlled by neural pathways unaffected by drugs administered intracerebroventricularly or because the cholinergic synapses activated by physostigmine do not carry the input from cold sensors.

Introduction

Administration of putative neurotransmitters to the cerebroventricular space is a technique used to study the influence of such substances on nerve pathways close to the ventricular wall. In an analysis of the central control of body temperature made with this technique, Bligh, Cottle & Maskrey (1971) found that intracerebroventricular (i.c.v.) injections of certain cholinomimetic drugs into sheep increased body temperature. Since the rise in body temperature was brought about in animals at low ambient temperature (t_a) by an increase in heat production, and in animals at high t_a by an inhibition of heat loss, it was proposed that the drugs were acting at cholinergic synapses in the neural path between cold sensors and heat production effectors at a point before the divergence of an inhibitory link to a path presumed to run between warm sensors and heat loss effectors. The reports by Maskrey (1971) that atropine injected into the cerebral ventricles of sheep exposed to cold inhibited shivering and that the responses elicited by i.c.v. carbachol or physostigmine could be blocked by prior i.c.v. injection of atropine supported the proposition that an atropine-sensitive cholinergic synapse exists in the neuronal chain linking the input from cold sensors with the output controlling heat production.

Cats respond similarly to sheep when given i.c.v. injections of those cholinomimetic drugs whose actions are blocked by atropine (Hall, 1972; Baird & Lang, 1973). In cats, i.c.v. administration of nicotine also elicits a thermoregulatory response, involving activation of heat loss mechanisms and a fall in body temperature. This has been taken to indicate that nicotinic receptors exist in the neural pathway controlling heat loss (Hall, 1972; Baird & Lang, 1973). The 'tremorgenic' drugs, tremorine and oxotremorine, have also been injected into the CNS of cats to induce a condition akin to Parkinsonism (Conner, Rossi & Baker, 1966a). These drugs elicit responses somewhat similar to those seen after injections of analogues of acetylcholine (ACh) when introduced into the cerebral ventricles, the hypothalamus or the caudate nucleus (Connor *et al.*, 1966a & b; Hall, 1972; Rudy & Wolf, 1972). Body temperature usually rises although the rise depends on drug dose and can be variable, and an increase occurs in involuntary muscular activity but whether this is 'tremor' or 'shivering' is uncertain (Rudy & Wolf, 1972).

The present experiments were carried out to gain further information about the nature of the sensitivity to cholinomimetic substances injected into the cerebroventricular space of sheep; the drugs nicotine and oxotremorine have been included to determine any related influence they may have on thermoregulatory control. A preliminary report of the experiments has been made (Johnson & Smith, 1974).

Methods

Experiments were done on nine castrated male Welsh Mountain sheep weighing between 31.6 and 52.5 kg. The animals were shorn every 3 weeks and kept indoors at 25 ± 3° C t_a to standardize the thermal stress. When experiments were done at neutral temperatures, animals were placed in a temperature controlled chamber maintained at 25° C t_a. For experiments in hot conditions the temperature in the chamber was raised to 40° C and for experiments in cold conditions it was lowered to 10° C, except when extreme cold was required when t_a was lowered to between −2° and +4° C.

Experiments usually lasted for 3-4 h and were begun in the morning 17 h after the animals were fed. Measurements were made of rectal (t_{re}), trunk skin and ear temperature with fine uncovered thermocouples, the output of each of which was registered on a potentiometric recorder every 36 seconds. Respiratory movements were sensed with a pneumatic belt and recorded for 30 s every 5 min; respiratory frequency was counted for periods of 10-20 seconds. Electromyograms (EMG) were detected from needle electrodes through the skin of the thigh and recorded for 30 s in every 5 minutes. The occurrence of shivering was also checked visually and, on the basis of such checks and the EMG records, shivering was scored numerically between zero (no shivering) and five (sustained vigorous shivering).

Injections or infusions were made into a lateral cerebral ventricle through a stainless steel cannula (Barton, Bligh & Sharman, 1968) implanted under general anaesthesia at least 1 week before the animal was first used in an experiment. The accurate placement of each cannula was confirmed by x-radiography. Only one drug was injected in each 3-4 h experiment unless the interaction of two drugs was being studied; then the injections were given 10 min apart. Recordings were made for 1 h before the first injection and for 2-3 h afterwards. Drug doses were related to body weight and administered in a volume of 0.005 ml/kg; the volume of drug solution injected was therefore approximately 0.2 ml and it was flushed into the ventricle with 0.2 ml isotonic saline. Drugs were dissolved in sterile physiological saline (0.9% w/v NaCl solution) which was then passed through a 0.22 μm Millipore filter before injection.

The drugs were atropine sulphate (B.D.H.), carbachol (carbamylcholine chloride, B.D.H.), physostigmine sulphate (B.D.H., eserine), nicotine hydrogen (+)-tartrate (B.D.H.), oxotremorine hydrochloride (May & Baker Research Laboratories), pempidine tartrate (May & Baker) and the quaternary methyl derivative of dopamine (3,4-dihydroxyphenylethyltrimethylammonium iodide, prepared by Dr J.D.M. Pearson, A.R.C. Babraham).

Control experiments were done for all drug treatments. To test whether the injection procedure itself elicited a response, 0.4 ml saline was given i.c.v. 1 h before injection of the drug in 96 experiments. To check for any response to injection of a volume of 0.8 ml, an initial injection of 0.4 ml saline was followed after 1 h by a second injection of 0.4 ml saline in 6 experiments. In a further 6 experiments injections of sodium sulphate 6 nmol/kg were given as controls for the injections of physostigmine sulphate 12 nmol/kg. In no control experiment was any significant change observed in the thermoregulation of the animal.

Results

The effects of each drug (except oxotremorine) were examined at four or more dose levels. Each dose was given to 2 animals but all four doses were not necessarily given to the same two animals. Values given in the text or figures are mean values from experiments on two animals.

Physostigmine

Physostigmine was injected in doses of 1.5, 3, 6 and 12 nmol/kg into the cerebral ventricles of 6 sheep held at 10°, 25° or 40° C t_a. The non-thermoregulatory behaviour of all animals was normal except when 12 nmol/kg physostigmine caused some animals to become restless and noisy for about 5 minutes. The responses to all doses of physostigmine were an inhibition of panting in

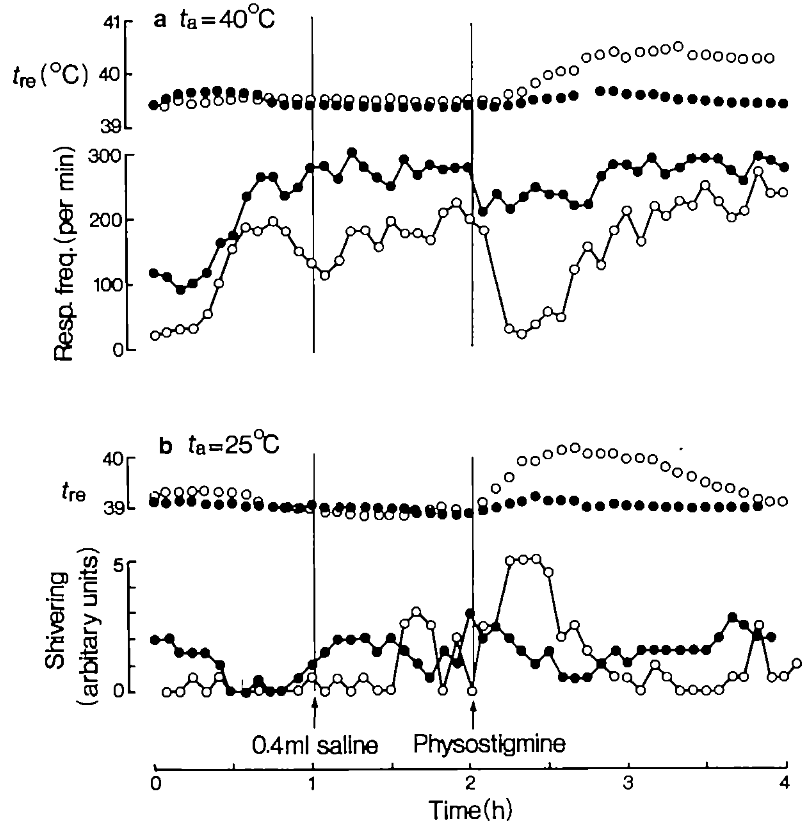

Figure 1 Effects of physostigmine 1.5 nmol/kg (●) or 12 nmol/kg (○) on (a) rectal temperature (t_{re}) and respiratory frequency at 40° C ambient temperature (t_a) and (b) t_{re} and shivering at 25° C t_a.

animals at 40° C t_a, an initiation of shivering in animals at 25° C and an increase in the intensity of shivering which already existed in animals at 10° C; in all animals t_{re} rose. Ear temperature was near t_a in all experiments and did not alter following injections of physostigmine. The shivering or respiratory responses and the changes in t_{re} which followed injections of the largest (12 nmol/kg) and smallest (1.5 nmol/kg) doses of physostigmine at 25° and 40° C t_a are shown in Figure 1. The rise in t_{re} which followed 3 nmol/kg physostigmine was intermediate between the rises recorded after 1.5 and 12 nmol/kg, but the rise after 6 nmol/kg was smaller than after 1.5 nmol/kg; why this response to 6 nmol/kg did not follow the dose-response relation for 1.5, 3 and 12 nmol/kg is not clear. At 10° C, the pattern of the response to physostigmine was similar to that at 25° C.

Carbachol

Carbachol was injected in doses of 0.7, 1.4, 2.7 or 5.5 nmol/kg into 6 sheep held at 10°, 25° and 40° C t_a. The respiratory and shivering responses observed were qualitatively similar to those seen after injections of physostigmine, although not as consistent. At 25° C t_a, the ear temperatures of some animals were near to t_{re}; following injections of carbachol the ear temperatures of these sheep decreased to near t_a indicating a peripheral vasoconstriction. The t_{re} rose by an average of 0.63° C within 1 h of injection in 19 of 24 experiments, but t_{re} was unchanged in 2 experiments and fell in 3 experiments. One of the animals used was inconsistent in its responses and the 3 experiments in which t_{re} decreased after carbachol injection were all done on this animal. However, even when the observations on this animal are disregarded, the rises in t_{re} measured following carbachol injections were not obviously correlated with the doses of carbachol administered.

Nicotine

Nicotine was injected i.c.v. into 4 sheep in single

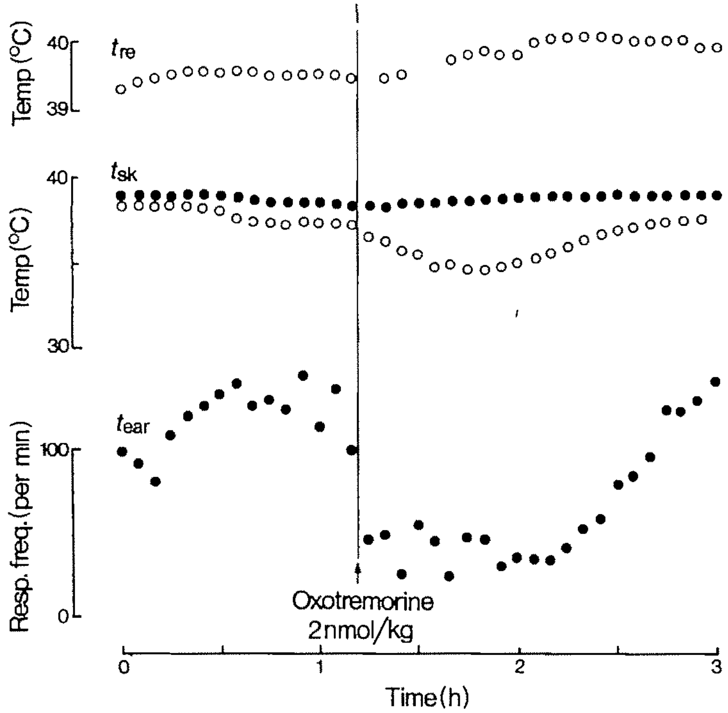

Figure 2 Effect of oxotremorine 2 nmol/kg on rectal (t_{re}), trunk skin (t_{sk}) and ear (t_{ear}) temperatures and respiratory frequency of sheep at 32° C ambient temperature.

doses ranging from 6 to 48 nmol/kg at 10°, 25° and 40° C t_a. No changes in t_{re} were observed following injections of doses of 24 nmol/kg or less, but after administration of 48 nmol/kg, animals became very restless for 15-20 min and during that period t_{re} rose. Continuous infusions of nicotine were made into the ventricles of 4 animals at dose rates between 2 and 6 nmol kg^{-1} min^{-1} for periods of 70-80 minutes. No thermoregulatory changes were observed and t_{re} increased by an average of only 0.3° C in that time.

The quaternary methyl derivative of dopamine is 3-12 times more effective than nicotine as a ganglionic stimulant in a number of tissue preparations (Cuthbert, 1964). In 8 experiments on 3 sheep, i.c.v. injections of this substance in doses ranging from 0.5 to 50 nmol/kg were completely ineffective in eliciting any thermoregulatory response or alteration in t_{re}.

Oxotremorine

Injections of 2 nmol/kg oxotremorine given into the cerebral ventricles of 2 sheep held at 25° C t_a caused an immediate fall in ear temperature, initiation of muscular activity indistinguishable from shivering and rises in t_{re} of 0.5° and 1.2° C in the two animals during the following 75 minutes. When the same dose of oxotremorine was given to 2 animals with moderately elevated respiratory frequencies held at 32° C t_a, ear temperature and respiratory frequencies both began to decrease immediately and t_{re} increased by 0.5° and 0.7° C in the 2 animals during the following 70 min (Figure 2).

Atropine

Atropine injected alone into the cerebroventricular system of 4 sheep in doses of between 1.25 and 10 nmol/kg at 10°, 25° and 40° C t_a caused neither consistent nor substantial changes in the thermoregulatory pattern. This lack of response was unexpected because Maskrey (1971) had reported that atropine inhibited shivering and decreased body temperature of sheep exposed to cold; further experiments were therefore done with atropine administered at higher doses to sheep at lower t_a, in which conditions Maskrey (1971) suggested the response was best observed. In 6 experiments, animals were injected i.c.v. with 10 or 20 nmol/kg atropine at t_a between −2° and +4° C. Throughout these experiments the animals continued to shiver strenuously, and even after the

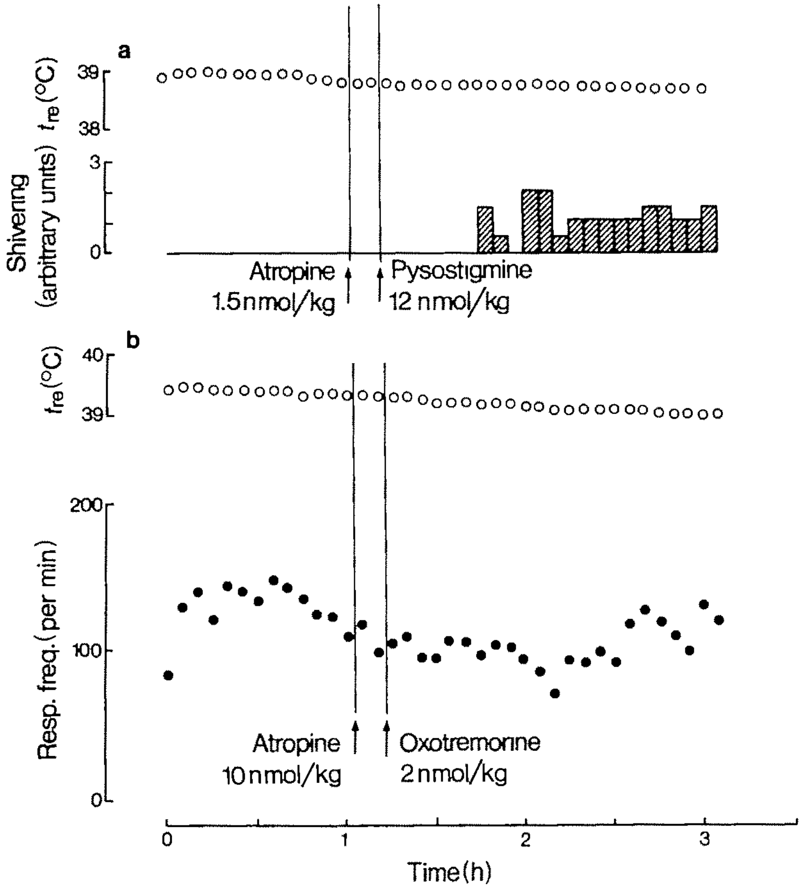

Figure 3 Inhibition by prior administration of atropine of the hyperthermic effects (a) of physostigmine given to sheep at 25° C ambient temperature (t_a) and (b) of oxotremorine given to sheep at 28° C t_a.

injection of 20 nmol/kg atropine i.c.v., t_{re} decreased by an average of only 0.26° C in 2 hours.

When atropine 12 nmol/kg was injected i.c.v. 10 min before physostigmine 12 nmol/kg, the initiation of shivering and rise in t_{re} usually seen after physostigmine injection no longer occurred. In successive experiments, the atropine dose preceding the physostigmine was reduced from 12 to 6, 3, 1.5 and 0.15 nmol/kg. An atropine dose of 1.5 nmol/kg completely blocked the hyperthermic effect of 12 nmol/kg physostigmine (Figure 3a) but an atropine dose of 0.15 nmol/kg did so incompletely. Atropine also provided a complete block of the thermoregulatory effects induced by oxotremorine. If atropine (10 nmol/kg, i.c.v.) was given 10 min before oxotremorine 2 nmol/kg, animals at 25° C t_a no longer began to shiver and at higher t_a the reduction in panting usually induced by i.c.v. oxotremorine no longer occurred (Figure 3b).

The effect of carbachol was only partially inhibited by doses of atropine which were completely effective against physostigmine and oxotremorine. Some shivering and a slight rise in t_{re} were recorded when carbachol 6 nmol/kg was injected 10 min after atropine 10 nmol/kg and these responses were even greater when only 5 nmol/kg atropine was given prior to the carbachol.

Pempidine

Pempidine is a long-acting ganglion-blocking agent which inhibits the peripheral nicotinic actions of cholinomimetic drugs (Corne & Edge, 1958). When injected in doses of between 3 and 24 nmol/kg into 4 sheep held at 10°, 25° or 40° C t_a, pempidine caused no change in thermoregulation and led to no change in t_{re}. When pempidine (24 nmol/kg i.c.v.) was injected 10 min

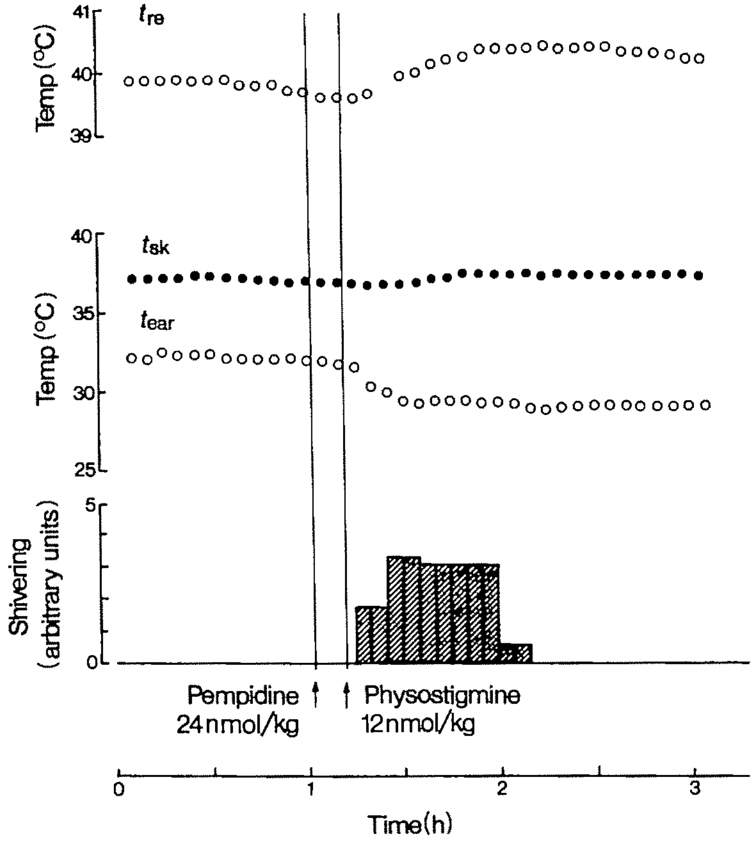

Figure 4 Failure of the ganglion-blocking drug, pempidine, to inhibit the rises in rectal temperature (t_{re}) and skin temperature (t_{sk}), fall in ear temperature and initiation of shivering following i.c.v. physostigmine given to sheep at 25° C ambient temperature.

before injections of physostigmine (12 nmol/kg) (Figure 4), carbachol (6 nmol/kg) or oxotremorine (2 nmol/kg) the inhibition of heat loss, accentuation of heat production and rise in t_{re} usually observed when these drugs were injected alone occurred apparently unaltered by the pretreatment with pempidine.

Discussion

The report of sensitivity to cholinomimetic drugs in the cerebroventricular space of sheep made by Bligh *et al.* (1971) is substantiated by the observations made here. Both the acetylcholine analogue, carbachol, and the anticholinesterase, physostigmine, inhibited panting of animals in the heat, caused vasoconstriction in the ears when this did not already exist and, in cool conditions, initiated or accentuated shivering. Since the physostigmine presumably had its effect by raising the levels of endogenous ACh, the response to both drugs can reasonably be attributed to stimulation of cholinergic synapses adjacent to the ventricular wall. The carbachol and physostigmine increased heat production at low t_a and decreased heat loss at high t_a; therefore, the cholinergic synapses might be considered to lie in the neural path by which heat production is controlled, at a point before the origin of a path inhibiting heat loss, as Bligh *et al.* (1971) suggested. Whether the same cholinergic synapses transmit information from cold sensors will be discussed later.

When nicotine was given in progressively increasing doses, no initiation or inhibition of thermoregulatory responses was ever observed. The highest single dose used (48 nmol/kg) caused animals to become restless for 15-20 min and t_{re} rose but this was the result of greater activity and not of a coordinated thermoregulatory effect. Continuous infusions of nicotine or injections of a powerful ganglionic stimulant, the quaternary methyl derivative of dopamine, were similarly ineffective, causing only restlessness when given in large doses. No receptors sensitive to i.c.v. nicotine therefore appeared to exist in the thermoregulatory control system of these sheep.

These observations are supported by the findings of complete, or almost complete, inhibition by atropine of the effects of intracerebroventricularly administered carbachol, physostigmine and oxotremorine, and the lack of effect of the ganglion-blocking drug, pempidine. Because no natural or induced thermoregulatory response was ever blocked by pempidine, one cannot be certain that an effective dose of this drug was used; but since pempidine 24 nmol/kg did not block the effect of physostigmine 12 nmol/kg whereas atropine 1.5 nmol/kg inhibited the response completely (Figure 3a), atropine must be at least 16 times more effective in blocking these cholinergic synapses than pempidine. In pharmacological terms, the sensitivity to cholinomimetic substances given into the lateral cerebral ventricles of sheep was muscarinic and not nicotinic.

The action of oxotremorine has frequently been related to the activity of ACh (e.g. George, Haslett & Jenden, 1966), and oxotremorine has been described as the most potent muscarinic agent known (Holmstedt, 1967). The similarity of the responses to oxotremorine and to other cholinomimetic drugs was, therefore, not surprising. From the present observations, there was no reason to believe that the response to oxotremorine was due to stimulation at a different site from that affected by physostigmine, carbachol and atropine. The muscular activity induced by i.c.v. injection of oxotremorine was not apparently different from shivering. Although details of the EMG were not studied closely, the muscular response to oxotremorine was similar, both in the EMG and visually, to that of sheep exposed to cold and to that induced by i.c.v. physostigmine or carbachol. Injections of oxotremorine which elicited an increased muscular activity at neutral t_a did so to a lesser extent or not at all in animals in warmer conditions; the rise in t_{re} in such conditions resulted from an inhibition of panting. Therefore, although there was no unequivocal identification of shivering and differentiation from tremor, the results indicated that, like other cholinomimetic drugs given i.c.v. in sheep, oxotremorine was affecting primarily thermoregulatory control rather than muscular tremor.

Although the observations made here confirm the report of Bligh *et al.* (1971) of cholinomimetic sensitivity in the cerebroventricular system of sheep, they are not entirely consistent with their proposal that the sensitivity is due to the presence of a cholinergic synapse in the neural pathway between cold sensors and heat production effectors. Physostigmine injected i.c.v. led to shivering and elevation of t_{re} presumably by allowing the level of endogenous ACh in some sensitive region to increase. Atropine, a competitive antagonist of Ach, completely inhibited the shivering and hyperthermia, presumably by counteracting this effect of endogenous ACh. However, atropine given alone in doses more than 10 times greater than required to block the action of physostigmine did not appear to change the intensity of shivering of animals exposed to cold, and t_{re} decreased only very slightly. Similar observations have been made on cats. Although 0.5 mg/kg atropine given intraperitoneally completely inhibited the tremor induced by carbachol injected into the caudate nucleus (Connor *et al.*, 1966b), larger doses of atropine (1-3 mg/kg i.p.) had no inhibitory effect on cold-induced shivering and led to no significant change in t_{re} (Stuart, George, Freeman, Hemingway & Price, 1961). An immediate deduction that the site of action of physostigmine, and possibly also of carbachol and oxotremorine, might not be on the cold sensor-heat production pathway is, however, only one of several explanations which can be offered for the observation.

One reason why cold-induced shivering was not depressed by doses of atropine which totally inhibited the response to i.c.v. physostigmine may be that shivering can be activated by a relatively small proportion of the periventricular neural pathways which are apparently involved in the control of heat production. If this were so, then i.c.v. injections of physostigmine, carbachol and oxotremorine could initiate shivering by stimulating these pathways, and atropine would block the effect. During exposure to cold, similar pathways either more distant from the site of injection or more remote from the ventricular wall might carry sufficient facilitatory information from peripheral cold sensors to initiate and maintain shivering despite the presence within the ventricles of a dose of atropine many times that necessary to block a stimulatory dose of a cholinomimetic drug.

A related explanation depends on the proposition that the main control of shivering may be exercised by structures quite outside the region affected by drugs added to the cerebroventricular space, possibly in the spinal cord. Evidence can be adduced for some extra-hypothalamic control of shivering, with only limited facilitation or inhibition arising from the hypothalamic region (Bligh, 1973). If, in the cold conditions used in the present experiments, only a limited facilitation of shivering was exerted by neurones adjacent to the cerebral ventricles, block of this small influence by

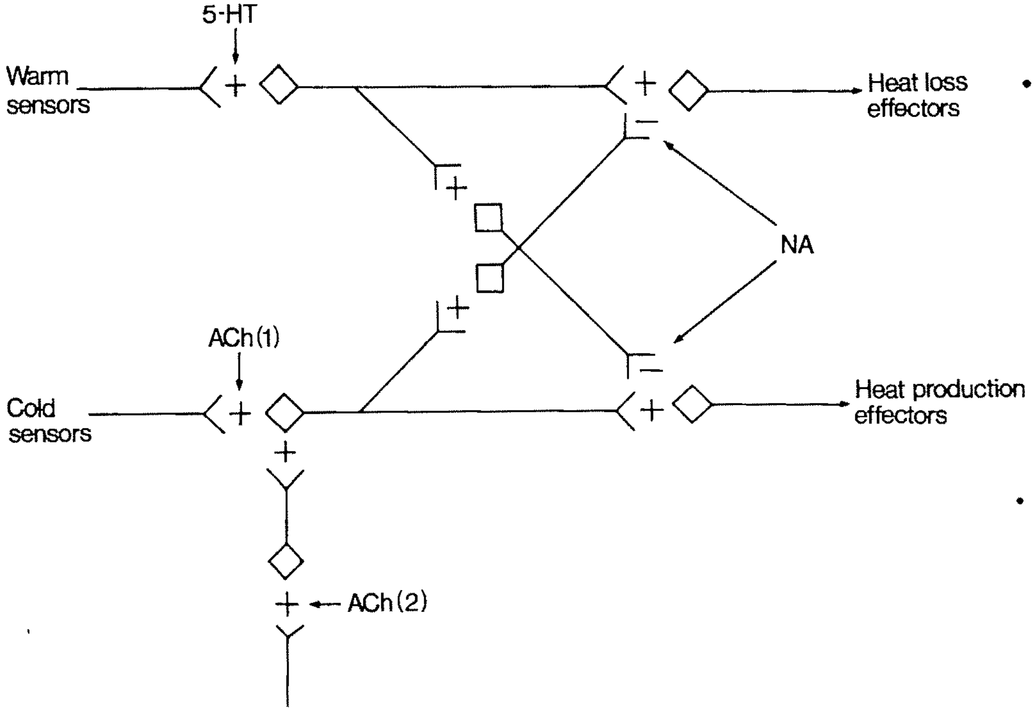

Figure 5 Neuronal model of the central control of body temperature in sheep, proposed by Bligh, Cottle & Maskrey (1971). Acetylcholine (ACh) (1) is the suggested site of a cholinergic synapse according to Bligh *et al.*; ACh (2) is a possible alternative site of a cholinergic synapse suggested by the present results. 5-HT = 5-hydroxytryptamine; NA = noradrenaline.

atropine may have passed undetected as a slight decrease in shivering and led to the observed small fall in core temperature. However, if such is the explanation, the thermoregulatory system proposed by Bligh *et al.* (1971) cannot be considered to be truly descriptive of the central control of body temperatures of sheep during exposure to cold.

A further possibility is that the cholinergic synapses activated by cholinomimetic drugs do not carry input signals from cold sensors. If the cholinergic synapses occurred, not in the input pathway from cold sensors as postulated by Bligh *et al.* (1971), but in an input converging onto the pathway between cold sensors and heat production effectors after the afferent input and before the divergence of the proposed crossed inhibition to the parallel pathway between warm sensors and heat loss effectors, the predicted behaviour of such a neuronal network would be consistent with all observations. In the format used by Bligh *et al.* (1971) the network would be represented as shown in Figure 5.

The report by Maskrey (1971) that i.c.v. atropine blocked shivering when given to cold-exposed sheep under conditions virtually identical to those used here remains unexplained by any of the proposals made above, and further experiments are needed to determine the repeatability of the response. A more detailed analysis is also required of the muscular activity elicited by i.c.v. injections of cholinomimetic drugs to determine whether it is tremor, shivering or a combination of the two. The fact that the muscular activity induced by i.c.v. cholinomimetic drugs in sheep is coordinated with changes in panting and peripheral vasomotor tone indicates strongly that the overall response is thermoregulatory and that the muscular activity is, at least partly, shivering. The general conclusion remains that, in sheep, atropine-sensitive cholinergic synapses apparently occur close to the ventricular wall in neural pathways involved in thermoregulatory control.

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THE EFFECTS OF CATECHOLAMINES AND ADRENOCEPTOR BLOCKING DRUGS ON THE CANINE PERIPHERAL LYMPH FLOW

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1 Blood flow through the femoral artery, lymph flow in a lymphatic vessel in the femoral triangle and metatarsal distal venous pressure were measured simultaneously in a canine moving hind limb.

2 Low intra-arterial doses of adrenaline and noradrenaline increased lymph flow even in the presence of marked arterial vasoconstriction. In contrast, isoprenaline increased arterial blood flow without affecting lymph flow rate.

3 Phenoxybenzamine, dihydroergotoxine, and nicergoline did not inhibit the lymphatic flow increase induced by adrenaline at doses active on arterial or venous vascular alpha-adrenoceptors.

4 Propranolol given intra-arterially into animals pretreated with alpha-adrenoceptor blocking agents restored the vasoconstrictor effect of adrenaline (reversal of adrenaline reversal).

Introduction

Adrenergic amines affect differently central and peripheral lymph flow. Adrenaline always increases *thoracic duct* lymph flow (Gesler, Matsuba & Dragstedt, 1956; Shim, Pollack & Drapanas, 1961; Doemling & Steggerda, 1962). Noradrenaline may decrease flow as a result of an increased thoracic duct contractility (Földi & Zoltàn, 1966). At doses which are hypertensive on arterial blood pressure, thoracic duct lymph flow may be increased (Wernze, Fujii & Sembach, 1965; Fujii & Wernze, 1966).

Renal lymph flow is increased by noradrenaline (Fujimoto & Lockett, 1970). *Hind limb flow* remains mainly unchanged after intra-arterial administration of doses of adrenaline sufficient to affect local venous blood outflow (Lewis & Winsey, 1970).

Whether these actions of catecholamines are direct or indirect is still an open question because many functions susceptible to catecholamines are related to lymph flow regulation.

Our experiments in the canine hind limb were designed to measure lymph flow simultaneously with local arterial blood flow and venous pressure, to study the interrelations between these functions. The effect of blocking the adrenoceptors of the lymphatic system was also investigated using alpha- and beta-adrenoceptor blocking drugs.

Methods

Experiments were carried out on male Beagle dogs anaesthetized with sodium pentobarbitone (30 mg/kg, i.v.). A constant rectal temperature of 37.5°C was maintained and cooling of the limb was prevented.

Blood pressure was recorded from the carotid artery through a polyethylene cannula connected to a Statham strain-gauge.

Venous pressure was measured with a Silastic cannula distally inserted into a small dorsal vein of the paw and connected to a Statham venous pressure transducer.

Femoral blood flow was estimated on the same leg by an electromagnetic blood flow-meter (Nycotron), the flow probe being placed on the femoral artery within the femoral triangle.

Femoral lymph vessels in the femoral triangle on the same side were dissected out and cannulated according to Stürmer (1966), to assess lymph flow.

Evans blue dye (0.5%) was injected i.m. into different areas of the leg to aid visualization of lymphatics. Constant basal lymph flow was assured by a passive mechanical movement of the leg according to Stürmer (1966) and recorded by a photoelectric drop recorder.

Drugs were administered into a systemic vein or into the femoral artery through a tributary artery arising distal to the flow probe. A Palmer syringe pump was used to infuse drugs or solvents at a constant rate of 0.4 ml/minute. Systemic blood pressure, venous pressure, lymph flow, and femoral blood flow were simultaneously and continually recorded on a galvanometric Honeywell Visicorder.

Vascular resistances were calculated as peripheral resistance units (PRU)

$$\mathrm{PRU} = \frac{\text{mean arterial blood pressure}}{\text{mean femoral blood flow}}$$

Drugs

These were, bradykinin triacetate trihydrate (Calbiochem), (±)-isoprenaline hydrochloride (Prodotti Gianni), (−)-adrenaline (Rhône-Poulenc), (−)-noradrenaline (Prodotti Gianni), (±)-propranolol (Prodotti Gianni), phenoxybenzamine HCl (a gift from SKF), nicergoline maleate (Sermion, Farmitalia) and dihydroergotoxine (DHE) as an equal part solution of dihydrocristine, dihydroergocryptine, dihydroergocornine as methansulphonates. The following solution was employed as placebo: NaCl 6 mg, methansulphonic acid 0.0357 mg, ethanol (96%) 2 mg, water up to 1 ml.

Results

Basal parameters were initially controlled for 1-2 h and animals with a lymphatic flow of less than 3-4 drops/min were discarded. Mean basal values of femoral blood flow, venous, and arterial blood pressure were respectively 80 ml/min, 10, and 130 mmHg.

Control

Vascular and lymphatic reactivity were subsequently tested by occluding the femoral artery with a pneumatic cuff for at least 1 min, and by injecting bradykinin (0.05-0.1 μg kg^{-1} min^{-1}) over 10 minutes. Removal of the arterial occlusion was followed by a marked increase in blood flow (reactive hyperaemia). During arterial occlusion, the lymph flow was stopped completely; a prompt return to control values was observed without any increase of lymph flow. Bradykinin administration increased femoral and lymph flows by 200 and 150% respectively.

The effect of catecholamines and adrenoceptor blocking agents (alone and combined) are described separately.

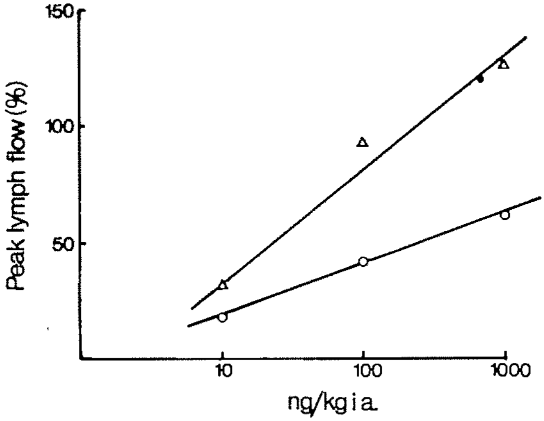

Figure 1 Peak responses of lymph flow in the hind limb to graded doses of adrenaline (○) and noradrenaline (△) given intra-arterially.

Local effects of catecholamines

Intra-arterial injections of adrenaline and noradrenaline increased lymph flow in all dogs (19 animals); the response was satisfactorilly related to the dose in the range, 0.01-1 μg/kg. Noradrenaline was slightly more active than adrenaline (see Figure 1). In contrast, isoprenaline (0.001 to 0.1 μg/kg) did not modify lymph flow. Adrenaline (0.01 μg/kg) produced a marked increased in arterial blood flow (Figure 2). In contrast, higher doses reduced flow down to zero, the effect being preceded by a short initial increase. Venous pressure was slightly decreased by adrenaline (0.01 and 0.1 μg/kg) but was consistently, but variably increased by 1 μg/kg intra-arterially. Increase in lymph flow was generally concomitant with a strong reduction of both blood flow and venous pressure. It preceded venous hypertension when present, i.e. at the dose of 1 μg/kg. Noradrenaline affected the cardiovascular parameters like adrenaline, whereas isoprenaline produced a parallel increase in both blood flow and venous pressure. Systemic arterial blood pressure was not affected by the low and medium doses of the catecholamines: adrenaline (1 μg/kg) produced small negative or positive variations. All the above effects were short-lasting.

Local effects of adrenoceptor blocking drugs

Adrenoceptor blocking drugs (ergotamine, phenoxybenzamine, nicergoline, DHE, and propranolol) were infused intra-arterially for 5-10 min to evaluate their local effects (see Figure 3). Ergota-

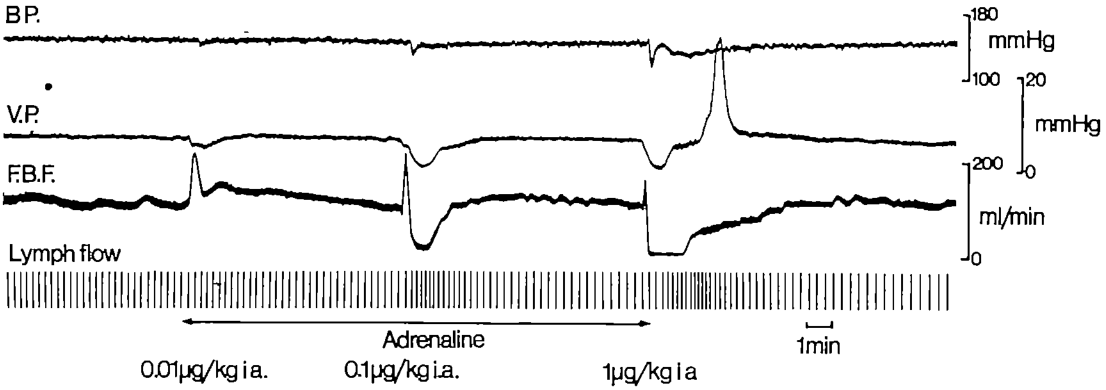

Figure 2 Anaesthetized dog: variations of systemic blood pressure (B.P.), metatarsal venous pressure (V.P.), femoral blood flow (F.B.F.), and lymph flow (recorded as drops) after intra-arterial injections of increasing doses of adrenaline.

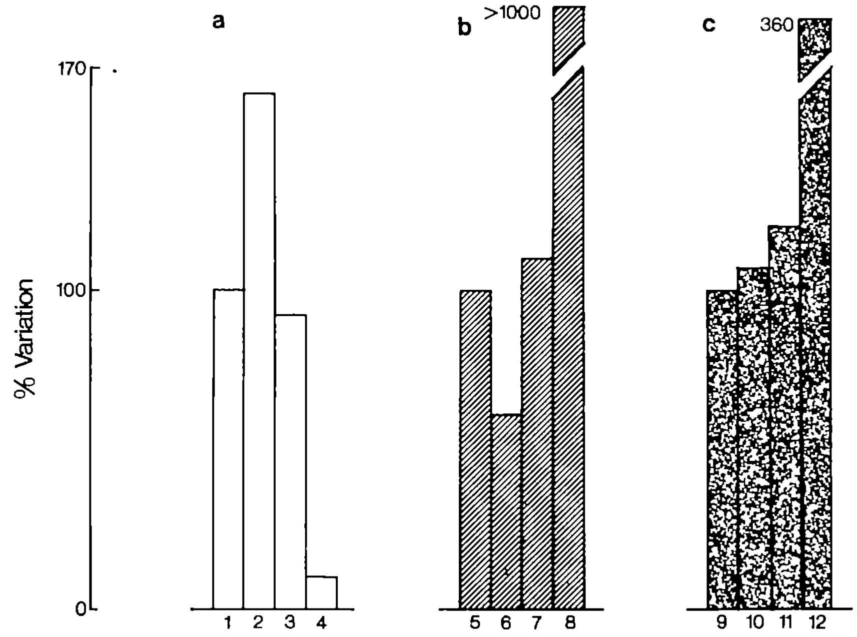

Figure 3 Mean percentage variations of femoral blood flow, vascular femoral resistance and venous pressure (from a metatarsal vein) during intra-arterial administration of nicergoline, dihydroergotoxine, and ergotamine. Mean percentage variations of (a) femoral blood flow, (b) vascular femoral resistance and (c) venous pressure (from a metatarsal vein) during intra-arterial administration of nicergoline (1 $\mu g\ kg^{-1}\ min^{-1}$; 2-6-10), dihydroergotoxine (1 $\mu g\ kg^{-1}\ min^{-1}$; 3-7-11) and ergotamine (1 $\mu g\ kg^{-1}\ min^{-1}$; 4-8-12), control values 1-5-9.

mine decreased femoral blood flow and increased venous pressure without arterial blood pressure modifications; these effects, present at 0.2 $\mu g\ kg^{-1}\ min^{-1}$, became marked at 1 $\mu g\ kg^{-1}\ min^{-1}$. Lymph flow was increased the first minutes of infusion and then decreased for a long period of time (over 1 hour).

DHE administered at 1 $\mu g\ kg^{-1}\ min^{-1}$, was completely ineffective, while at 5 $\mu g\ kg^{-1}\ min^{-1}$ it decreased femoral blood flow by 40% and increased venous pressure by 50% without modification of lymph flow. All these effects were long-lasting. The long-lasting DHE vasoconstrictor effect was sometimes preceded by a short-lasting

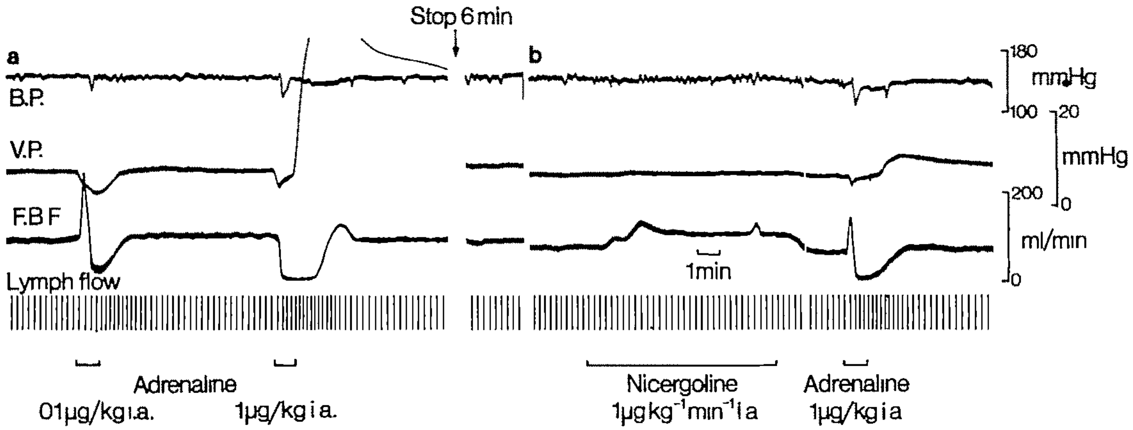

Figure 4 Anaesthetized dog: variations of systemic blood pressure (B.P.), metatarsal venous pressure (V.P.), femoral blood flow (F.B.F.), and lymph flow (recorded as drops). (a): Effect induced by adrenaline, 0.1 and 1 μg/kg. (b) Effect of adrenaline (1 μg/kg) after intra-arterial infusion of nicergoline

vasodilatation which was also observed after administering its solvent alone.

At a constant infusion rate of 1-5 μg kg^{-1} min^{-1} nicergoline (Arcari, Dorigotti, Fregnan & Glässer, 1968) produced marked increases (60 and 115% respectively) in femoral blood flow, which lasted throughout the administration time, returning to control level as soon as the infusion was completed. Arterial blood pressure and lymph flow were not modified. A slight (<10%) and brief increase in venous pressure was observed after administration of 5 μg kg^{-1} minute^{-1}. Phenoxybenzamine at a constant infusion rate of 5 and 10 μg kg^{-1} min^{-1} did not affect arterial or venous pressure or lymph flow, while it had a weak action on peripheral blood flow, which increased during the administration period with a quick return to near control values when the infusion was over. Intra-arterial administration of propranolol (10, 100 and 1000 μg/kg) did not modify the above parameters.

Adrenaline: effects on animals pretreated with alpha- and beta-adrenoceptor blocking drugs

In this set of experiments a standard dose of adrenaline (1 μg/kg) was injected intra-arterially 2, 5 and 30 min after the administration of phenoxybenzamine, DHE, nicergoline, and propranolol. Combined pretreatments of phenoxybenzamine-propranolol, and DHE-propranolol were also tested. Ergotamine was not used as an alpha-adrenoceptor blocking drug because of its marked and long-lasting intrinsic effects on the above functions. Neither DHE, (1-5 μg kg^{-1} min^{-1}) nicergoline, (1-5 μg kg^{-1} min^{-1}) (Figure 4), phenoxybenzamine (5 and 10 μg kg^{-1} min^{-1}) nor propranolol (10, 100 and 1000 μg/kg) modified the increase in lymph flow to adrenaline. The venous hypotensive phase induced by adrenaline remained practically unchanged in the animals treated by alpha-adrenoceptor blocking drugs. In contrast, venous hypertension was abolished, or at least reduced up to 80%, by DHE, phenoxybenzamine, and nicergoline. In addition, DHE and phenoxybenzamine converted the reduction in arterial blood flow into a clear-cut increase (adrenaline reversal). Nicergoline reduced the intensity and duration of adrenaline vasoconstriction. In control experiments, the highest intra-arterial dose of propranolol reduced the increase in arterial flow to isoprenaline by at least 60%. The increase of blood flow induced by adrenaline in animals pretreated with DHE and phenoxybenzamine was converted into a small decrease of flow by administering propranolol, 10 or 100 μg/kg intra-arterially (Figure 5); also the adrenaline hypertensive effect on venous pressure was restored (reversal of adrenaline reversal).

Discussion

The experiments were performed to study the effects of catecholamines and adrenoceptor blocking agents (alone and combined) on the lymphatic system of the hind limb of the dog. Our results clearly show that only adrenaline and noradrenaline increase lymph flow when injected intra-arterially at doses inactive on systemic arterial blood pressure.

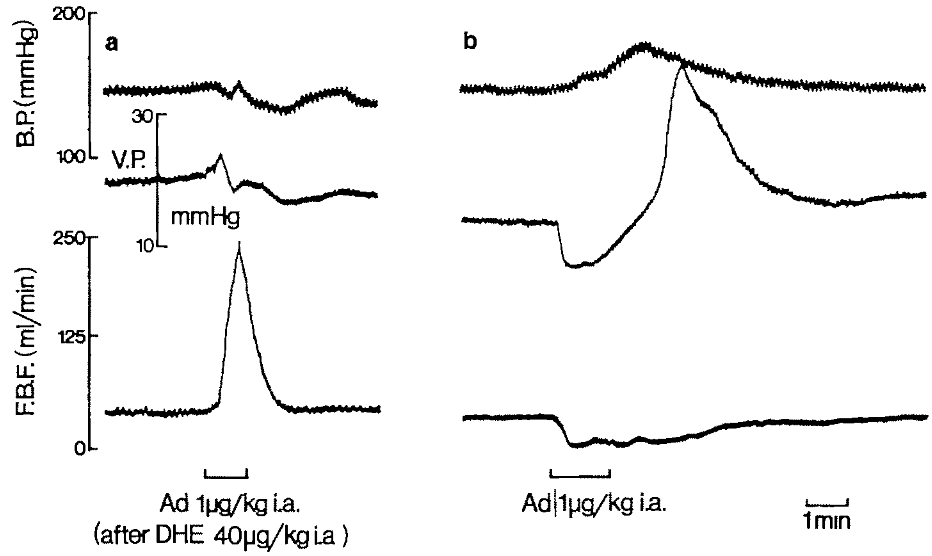

Figure 5 Anaesthetized dog: variations of systemic blood pressure (B.P.), metatarsal venous pressure (V.P.), and femoral blood flow (F.B.F.). (a). Adrenaline (Ad) femoral vasodilatation in dihydroergotoxine (DHE) pretreated animal (adrenaline reversal). (b). Return to the previous vascular effects of adrenaline following propranolol, 10 µg/kg, i.a. (reversal of adrenaline reversal) 30 min previously.

These effects might be due to a direct action of adrenaline and noradrenaline on lymphatic vessels; in fact, they are independent of blood flow changes and of venous hypertension. Lymph flow is also increased by adrenaline when its vasoconstrictor effect is abolished by alpha-adrenoceptor blocking agents. An increase in blood flow induced in various ways (isoprenaline, reactive hyperaemia or nicergoline) does not influence lymph flow *per se*. On the other hand, a reduction of arterial blood flow does not produce an increase in lymph flow; occlusion of the femoral artery immediately stops lymph movement.

Venous hypertension might be excluded as a causal factor of the lymph flow increase since α-adrenoceptor blocking agents abolish venous hypertension without influencing the increase in lymph flow induced by adrenaline and noradrenaline.

The stimulatory activity of adrenaline and noradrenaline together with the lack of effect of isoprenaline, a pure beta-adrenoceptor stimulant, suggest that α-adrenoceptors are present in the lymphatic vessels of the canine hind limb.

In accordance with this hypothesis, noradrenaline was more active than adrenaline. Contrary to our expectation, alpha-adrenoceptor blocking agents did not antagonize the effects of adrenaline and noradrenaline even when given locally at doses sufficient to invert the adrenaline vasoconstriction to a clear-cut vasodilation (adrenaline reversal).

The observations provide further information about the interaction of alpha- and beta-adrenoceptor blocking agents. Smith & Nash (1969) showed that some beta-blocking drugs may restore to near normal values, the systemic blood pressure response to noradrenaline in phenoxybenzamine-treated animals. The results clearly show that a similar phenomenon can be observed also locally in the canine hind limb vasculature. Propranolol converted the vasodilator effect of adrenaline (in DHE and phenoxybenzamine-treated animals) to a vasoconstriction similar to the one displayed before alpha-adrenoceptor blockade.

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THE EFFECTS OF MORPHINE ON THE RELEASE OF NORADRENALINE FROM THE CAT ISOLATED NICTITATING MEMBRANE AND THE GUINEA-PIG ILEUM MYENTERIC PLEXUS-LONGITUDINAL MUSCLE PREPARATION

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1 Electrical field stimulation of either the cat isolated nictitating membrane or the guinea-pig ileum myenteric plexus-longitudinal muscle preparation caused the release of noradrenaline into the bathing medium.

2 In the cat nictitating membrane, the output per pulse of noradrenaline was constant at frequencies of stimulation from 0.5 to 15 Hz. In the guinea-pig myenteric plexus preparation the output per pulse of noradrenaline increased as the frequency of stimulation was increased from 2 to 16 Hz.

3 Phenoxybenzamine (29.3 µM) caused a marked increase in the noradrenaline output from both the cat nictitating membrane and guinea-pig myenteric plexus preparations.

4 Morphine (0.13-8 µM) inhibited the contractions of the cat nictitating membrane caused by electrical stimulation. This effect was greater at low (1 Hz) than at high (15 Hz) frequencies of stimulation. The site of action is at the nerve-smooth muscle junction.

5 The action of narcotic analgesic drugs on the cat nictitating membrane showed stereospecificity. Naloxone (0.1 µM) reversed the inhibition caused by normorphine (3.2 µM).

6 Morphine (3 µM) reduced the noradrenaline output from the cat nictitating membrane stimulated at 1 Hz but not at 15 Hz. At 1 Hz, the inhibition of noradrenaline output by normorphine (5 µM) was reversed by naloxone (0.25 µM).

7 Morphine (1.5 µM) did not alter the noradrenaline output from the guinea-pig myenteric plexus preparation stimulated at 2 or 16 Hz.

Introduction

Morphine has a very selective action on the peripheral autonomic system and only affects certain neuro-effector junctions. Thus in the adrenergic system, morphine inhibits contractions elicited by nerve stimulation of the cat nictitating membrane both *in vivo* and *in vitro* (Trendelenburg, 1957; Thompson, 1960; Cairnie, Kosterlitz & Taylor, 1961) and of the mouse vas deferens (Henderson, Hughes & Kosterlitz, 1972a). The action of morphine in these tissues is a specific effect and appears to involve an inhibition of noradrenaline release (Cairnie *et al.,* 1961; Henderson *et al.,* 1972a). Except in very high concentrations (>10 µM) morphine does not affect the release of noradrenaline from the rabbit isolated heart (Montel & Starke, 1973), vas deferens and portal vein (Hughes, unpublished observations). Why transmission is impaired at only certain synapses is unknown.

The purpose of this investigation was to examine the release of noradrenaline in the cat nictitating membrane at different frequencies of stimulation and the effects of morphine on the relationship between frequency and release. A parallel study has been made on the output of noradrenaline from the guinea-pig ileum myenteric plexus-longitudinal muscle preparation where it is known that morphine inhibits the release of acetylcholine (Paton, 1957; Schaumann, 1957; Cowie, Kosterlitz & Watt, 1968). Some of the results have already been reported to the British Pharmacological Society (Henderson, Hughes & Thompson, 1972b).

Methods

Cat nictitating membrane

Male or female cats of between 2 and 4 kg were anaesthetized by intraperitoneal injection of either a solution containing urethane (500 mg/kg) and chloralose (50 mg/kg) or a solution of sodium pentobarbitone (40 mg/kg). The medial smooth muscle of the nictitating membrane attached to a portion of cartilage was isolated by the method of Thompson (1958). The tissue was mounted in a 5 ml organ bath containing Krebs solution at 37° C and placed under 1 g tension.

Guinea-pig ileum myenteric plexus-longitudinal muscle

Male guinea-pigs weighing 300-600 g were killed by breaking the neck and exsanguination. A suitable length of ileum was removed and placed in warm Krebs solution; the terminal 10 cm of ileum was not used. The method of dissection of the myenteric plexus-longitudinal muscle preparation was a modification of that reported by Ambache (1954) and is described in full by Kosterlitz, Lydon & Watt (1970). The tissue was mounted in a 3 ml organ bath under 0.5 g tension.

Contractions of both the isolated nictitating membrane and the myenteric plexus-longitudinal muscle were recorded isometrically and displayed on a pen oscillograph. Electrical field stimulation was used to excite the tissues. Platinum electrodes were fixed vertically on opposite sides of the tissue and supramaximal (1.3 x maximal current) stimuli, 1.0 ms rectangular pulses were used throughout.

Determination of released noradrenaline

Biological and fluorimetric assay methods were used. Superfused spiral preparations of rabbit aortic and iliac arteries were used for the bioassay (Hughes, 1972). After stimulation, the fluid surrounding the nictitating membrane or myenteric plexus (donor tissues) was left for 3 min to permit the released noradrenaline to diffuse from the tissue into the surrounding bathing medium. The bath fluid was then transferred to a cascade system perfusing the assay tissues. The output of transmitter from the donor tissue was determined by bracketing contractions of the assay tissues to the released material with contractions elicited by standard doses of noradrenaline.

In several experiments the noradrenaline which diffused from the tissue into the surrounding bathing fluid after electrical stimulation was estimated fluorimetrically. The fluid was collected in cooled flasks containing disodium edetate (10 mg/ml bath fluid). After adjusting the pH to 8.6, the noradrenaline was adsorbed on alumina columns. After elution with 0.15 M perchloric acid the noradrenaline content was determined fluorimetrically by a modification of the method of O'Hanlon, Campuzano & Horvath (1970) as described by Hughes (1972). Standard amounts of noradrenaline were taken through this procedure in order to determine the total recovery of the amine.

The donor tissues were stimulated at 20 min intervals. Experiments in which the output of noradrenaline from the donor tissues declined by more than 10% per stimulus train were either rejected or further controls were obtained until the outputs were within 10% of each other. In any one experiment the output of noradrenaline after stimulation at a single frequency is the mean of at least two observations.

Electrical stimulation did not produce any destruction of noradrenaline; this was shown after removal of the donor tissues, when 100 pulses at 0.2 or 15 Hz were passed through the bathing fluid containing 0.33 ng/ml of noradrenaline. Bioassay showed that there was no loss of noradrenaline activity.

Endogenous noradrenaline content

The noradrenaline contents of the nictitating membrane and of the myenteric plexus-longitudinal muscle preparation were estimated by fluorimetric assay. After homogenization at 4° C with 4 ml 0.4 M perchloric acid, 0.2 ml 10% (w/v) EDTA solution and 0.2 ml Tris buffer (1 M, pH 8.6), the homogenate was centrifuged for 15 min at 15,000 *g*. The supernatant was concentrated over alumina columns and the noradrenaline content assayed fluorimetrically as described above.

Drugs and solutions

The bathing fluid was a modified Krebs solution of the following composition (mM): NaCl 118, KCl 4.75, $CaCl_2$ 2.54, $MgSO_4$ 1.19, $NaHCO_3$ 24, KH_2PO_4 0.93, glucose 11, tyrosine 0.25, ascorbic acid 0.1 and disodium edetate 0.027; it was bubbled with 95% oxygen and 5% carbon dioxide. The drugs used were dextrorphan tartrate (Roche Products), histamine acid phosphate (B.D.H.), 5-hydroxytryptamine-creatine sulphate (B.D.H.), hyoscine hydrobromide (Macfarlan Smith), levorphanol tartrate (Roche Products), morphine hydrochloride (Macfarlan Smith), naloxone hydrochloride (Endo Laboratories), (−)-noradrenaline bitartrate (B.D.H.), normorphine hydrochloride (Dr E.L. May), phenoxybenzamine hydrochloride (Smith, Kline & French), and phentolamine hydrochloride (Ciba).

A time interval of 20 min was used between exposures of the tissues to successive doses of narcotic analgesics to prevent the occurrence of tachyphylaxis.

Definition of terms

Output of transmitter This term is used to describe the amount of noradrenaline which diffuses from the stimulated tissue into the surrounding bathing fluid. This is not equal to the amount released from nerve endings but is assumed to be proportional to the actual amount released.

Fractional noradrenaline output per pulse This indicates the output of transmitter per pulse as a fraction of the total tissue content of noradrenaline determined after completion of the experiment.

Results

Identification of material released by electrical stimulation

When the assay tissues were superfused with phentolamine (160 nM), contractions to standard doses of noradrenaline and to the material present in the bath fluid after electrical stimulation of the donor tissue were reduced equally whereas contractions elicited by equiactive doses of histamine and 5-hydroxytryptamine were not decreased.

Noradrenaline output from the cat isolated nictitating membrane

After stimulation with trains of 100 pulses, the fractional noradrenaline output per pulse from the untreated nictitating membrane was not significantly different at 0.2, 1 and 15 Hz (Table 1). Incubation of the tissue for 1 h with phenoxybenzamine resulted in a 10 to 25-fold increase in the noradrenaline output at the three frequencies of stimulation. After phenoxybenzamine the noradrenaline output was slightly higher at 16 Hz than at 0.2 Hz but this rise with frequency was much smaller than that found in the rabbit vas deferens or portal vein (Hughes, 1972; Hughes & Roth, 1974).

Noradrenaline output from the guinea-pig myenteric plexus-longitudinal muscle

In this tissue stimulation with trains of 240 pulses at 2 and 16 Hz produced a frequency-output relationship (Table 1) different from that observed in the cat nictitating membrane. In the untreated

Table 1 Effects of phenoxybenzamine on fractional noradrenaline output from the nictitating membrane of the cat and the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum

Treatment	*Fractional noradrenaline output from nictitating membrane (x 10^5) at*			*Differences (0.2 vs 1 or 15 Hz)*	*Fractional noradrenaline output from myenteric plexus (x 10^5) at*		*Difference between frequencies*
	0.2 Hz	*1 Hz*	*15 Hz*		*2 Hz*	*16 Hz*	
None	0.16 ±0.03	0.34 ±0.11	0.24 ±0.06	+ 0.18 ± 0.11 (1 Hz) + 0.08 ± 0.04 (15 Hz)	1.81 ±0.29	3.52 ±0.70	1.71** ±0.51
Phenoxybenzamine (29.3 μM for 1 h)	4.8 ±1.4	5.3 ±1.2	6.4 ±1.1	+ 0.50 ± 0.61 (1 Hz) + 1.57 ± 0.50* (15 Hz)	15.3 ±3.5	26.1 ±2.7	10.8*** ±2.1

Each of 3 preparations of the nictitating membrane were stimulated at three frequencies, in the absence or presence of phenoxybenzamine. There were 12 control and 7 treated preparations of the myenteric plexus. The values are the means ± s.e.mean of the fractional output per pulse caused by trains of 100 pulses to the nictitating membrane and trains of 240 pulses to the myenteric plexus. The standard errors of the differences between the means were determined by paired analysis.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

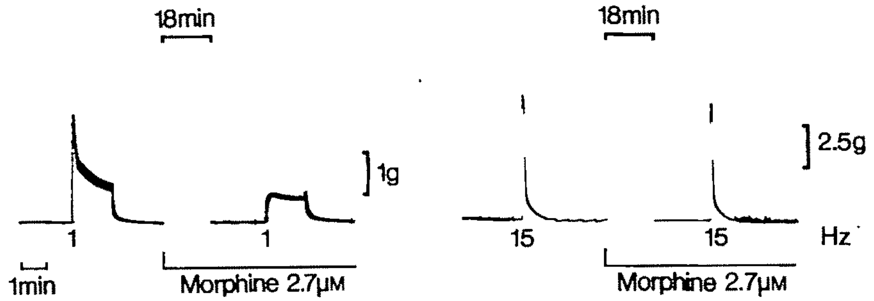

Figure 1 Effect of morphine (2.7 μM) on contractions of the cat isolated nictitating membrane stimulated at 1 and 15 Hz with trains of 100 pulses. These results were repeated in four experiments.

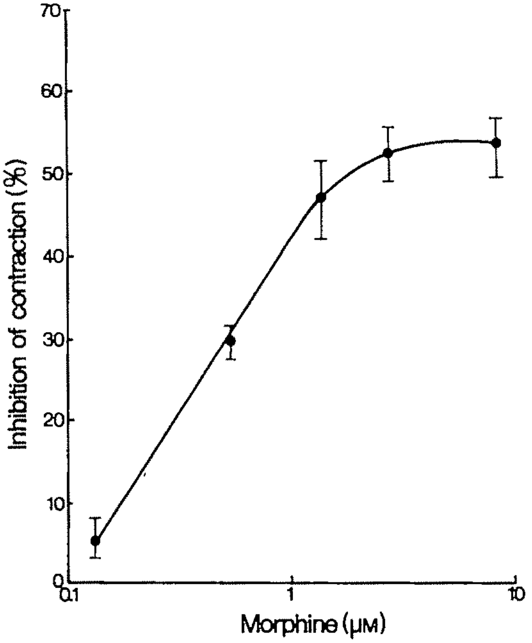

Figure 2 Effect of morphine on contractions of the cat isolated nictitating membrane stimulated at 1 Hz with trains of 30 pulses every 3 minutes. Mean results from 6 preparations. Vertical bars show s.e.mean. Ordinates: inhibition of contraction as a percentage of the initial response. Abscissae: morphine concentration (μM).

preparation the noradrenaline output per pulse at 16 Hz was approximately twice that at 2 Hz. Although, after treatment with phenoxybenzamine (29.3 μM), the noradrenaline outputs at each frequency were increased 8-fold there was still a 2-fold difference in the outputs per pulse at 2 Hz and 16 Hz.

Effect of morphine on contractions of the cat isolated nictitating membrane

Cairnie *et al.* (1961) found that morphine was more effective in reducing the size of the contractions at low frequencies than at high frequencies of stimulation in the cat nictitating membrane *in vivo.* A similar result was obtained in the *in vitro* preparation in which morphine (3 μM) produced a greater inhibition of the contractions due to stimulation at 1 Hz than at 15 Hz (Figure 1).

Morphine produced a dose-dependent inhibition of the contractions of the nictitating membrane stimulated at 1 Hz (Figure 2). The maximal inhibition was 50-60%, the residual contraction was unaffected by hyoscine (1.2 μM) but abolished by either bretylium (12 μM) or phentolamine (310 μM). The concentration of morphine required to produce 50% of the maximal inhibition (ED_{50}) was 0.5 μM. When the contractions due to stimulation at 1 Hz were reduced by normorphine (3.2 μM), which is equiactive with morphine in the guinea-pig ileum (Kosterlitz, Lord & Watt, 1972) and mouse vas deferens (Hughes, Kosterlitz & Leslie, 1974), naloxone (100 nM) completely reversed the inhibition of contraction (Figure 3). Levorphanol (3.2 μM) depressed the responses of the cat nictitating membrane whereas an equal concentration of dextrorphan (3.2 μM) did not. A higher concentration of dextrorphan (32 μM) did depress the response to stimulation, but this was not reversed by naloxone (1.3 μM) whereas the depression due to an equiactive dose of levorphanol was reversed by naloxone (300 nM).

Effect of morphine on the noradrenaline output from the cat nictitating membrane

The noradrenaline outputs from this tissue stimulated with trains of 100 pulses at 1 and 15 Hz were determined in normal Krebs solution and in Krebs solution containing morphine (3 μM) (Table 2). Since there was considerable variation in the noradrenaline output of different preparations, the results were obtained from paired observations. Morphine produced a 60% decrease in the

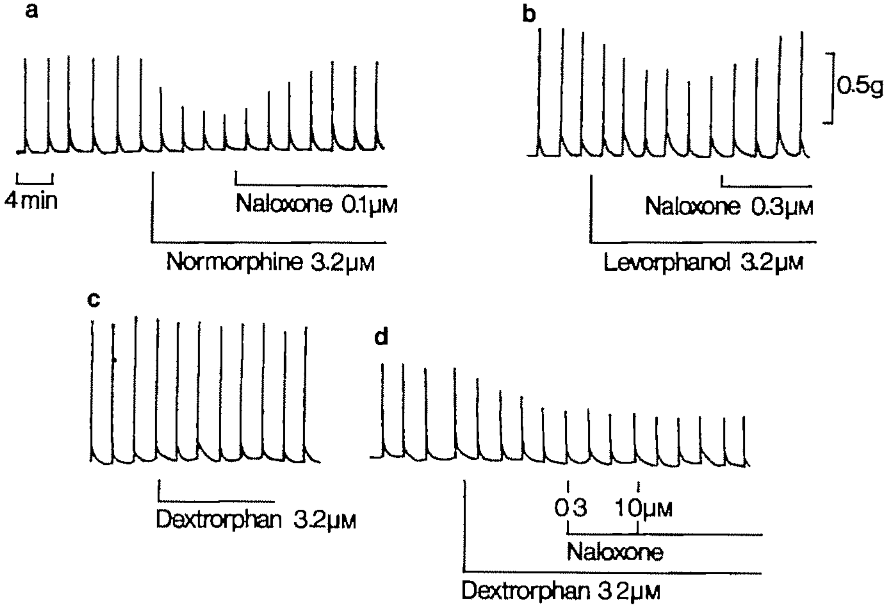

Figure 3 Effect of normorphine, levorphanol and dextrorphan on contractions of the cat isolated nictitating membrane stimulated at 1 Hz with trains of 10 pulses every 3 minutes. (a) Inhibition of contraction by normorphine (3.2 μM) was reversed by naloxone (0.1 μM). (b) Inhibition of contraction by levorphanol (3.2 μM) was reversed by naloxone (0.3 μM). (c) and (d) Dextrorphan (3.2 μM) did not inhibit the contractions; the inhibition of contraction produced by dextrorphan (32 μM) was unaffected by naloxone (1.3 μM). These results were repeated in three experiments.

Table 2 Effects of morphine on fractional noradrenaline output from the nictitating membrane of the cat and the myenteric plexus-longitudinal muscle preparation from the guinea-pig ileum

Treatment	*Fractional noradrenaline output from nictitating membrane (x 10^5) at*		*Difference for frequencies*	*Fractional noradrenaline output from myenteric plexus (x 10^5) at*		*Difference for frequencies*
	1 Hz	*15 Hz*		*2 Hz*	*16 Hz*	
None	0.86 ±0.07	0.92 ±0.29	+0.052 ±0.272	0.94 ±0.23	1.83 ±0.27	+0.89* ±0.35
Morphine	0.28 ±0.06	1.03 ±0.29	+0.74* ±0.24	1.28 ±0.31	1.94 ±0.14	+0.66 ±0.34
Difference for treatment	−0.58** ±0.047	+0.11 ±0.043		+0.34 ±0.38	+0.11 ±0.30	

Each of 4 preparations was stimulated at the two frequencies, in the absence and presence of morphine (nictitating membrane, 3 μM; myenteric plexus, 1.5 μM). The values are the means ± s.e.mean of the fractional output per pulse caused by trains of 100 pulses to the nictitating membrane or trains of 240 pulses to the myenteric plexus. The standard errors of the differences between the means were determined by paired analysis.
* $P < 0.05$, ** $P < 0.01$.

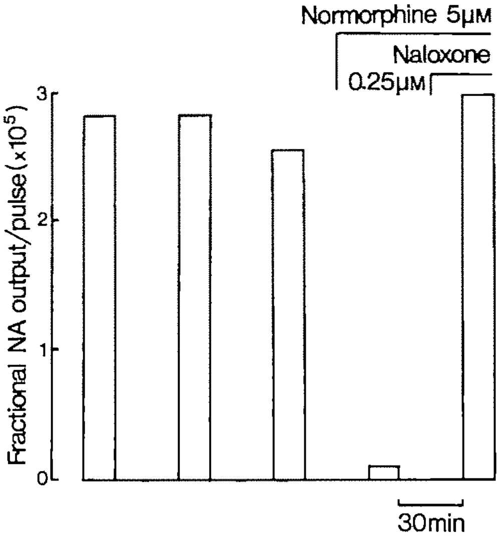

Figure 4 Reversal of normorphine inhibition of noradrenaline output by naloxone. Each column represents the noradrenaline output from the cat isolated nictitating membrane stimulated at 1 Hz with 240 pulses. Tissue pretreated for 1 h with phentolamine (36 μM). Ordinates: noradrenaline output (fractional noradrenaline (NA) output per pulse). Abscissae: time between stimulation periods.

output of noradrenaline at 1 Hz whereas the noradrenaline output at 15 Hz was not significantly altered. This greater effect of morphine on noradrenaline output at the lower frequency of stimulation correlates well with the greater depressant effect of morphine on the contractions (Figure 1).

After treatment of the nictitating membrane with phentolamine (36 μM), the noradrenaline outputs were determined after stimulation with trains of 100 pulses at 1 Hz applied every 30 minutes. Exposure of the tissue to normorphine (5 μM) for 15 min before stimulation resulted in a decrease in the noradrenaline output (Figure 4). When the tissue was subsequently treated with normorphine (5 μM) and naloxone (0.25 μM) for 15 min, the noradrenaline output returned to control levels.

Effect of morphine on the noradrenaline output from the myenteric plexus-longitudinal muscle preparation

After exposure of the tissue to morphine (1.5 μM) for 5 min the output of noradrenaline at 2 and 16 Hz was not significantly altered from control values (Table 2). Therefore, unlike the cholinergic innervation of the myenteric plexus, the adrenergic innervation is not sensitive to morphine.

Discussion

The inhibition of noradrenaline output from the cat nictitating membrane by morphine is due to a stereospecific effect at a morphine receptor site because the inhibitory effects are reversed by naloxone, and levorphanol has a much greater depressant effect than its dextro-isomer, dextrorphan. Since the concentrations of morphine required for this effect do not affect noradrenaline uptake into peripheral (Montel & Starke, 1973) or central neurones (Ciofalo, 1972), morphine would appear to act on the noradrenergic nerve terminals to decrease transmitter release.

The nictitating membrane may be added to the relatively few tissues with autonomic innervation in which a specific morphine receptor has been demonstrated. In the myenteric plexus of the guinea-pig ileum, the rabbit vagus-sinoauricular node junction (Kosterlitz & Taylor, 1959; Kennedy & West, 1967) and the mouse vas deferens, the possibility has not been excluded that morphine receptors may be present at sites other than the nerve-smooth muscle junction. On the other hand, the smooth muscle cells of the nictitating membrane are innervated by fibres which have their cell bodies in the superior cervical ganglion and no ganglion cells have been found within the smooth muscle (Gardiner, Hellmann & Thompson, 1962). Therefore, the inhibitory effect of morphine on noradrenaline release would appear to be due to an action on the nerve terminals innervating the smooth muscle.

The mechanism of noradrenaline release from the morphine-sensitive nictitating membrane differs in certain aspects from that in morphine-insensitive tissues such as the rabbit vas deferens and portal vein and the guinea-pig myenteric plexus. In the latter tissues, the output of noradrenaline per pulse increases at least 10-fold with a rise in frequency of stimulation from 0.5 to 16 Hz (Hughes, 1972, Hughes & Roth, 1974) while in the nictitating membrane there is only a 1.3-fold increase over a frequency range of 0.2 to 15 Hz. Although phenoxybenzamine increases the output of noradrenaline, it has little effect on the relationship of frequency to noradrenaline output in the tissues mentioned above. This observation would appear to exclude the possibility that neuronal and extraneuronal uptake (Hughes, 1972) or post-junctional or pre-junctional (Starke, 1972) α-adrenoceptors play any major role in determining this relationship. In this connection it should be emphasized that the selection of experimental conditions is of great importance, since the fractional output increases with increasing train length until a rapid decline sets in when the train length exceeds 400-500 pulses (Hughes & Roth, 1974); moreover, long periods of stimulation deplete the noradrenaline in the tissue stores irreversibly.

The greatest difference in noradrenaline output per pulse between the nictitating membrane and the other tissues is found at frequencies below 0.5 Hz. In the presence of phenoxybenzamine, the fractional output from the nictitating membrane is at these low frequencies similar to that at high frequencies ($5\text{-}6 \times 10^{-5}$) whereas in the rabbit vas deferens the fractional output falls from 1×10^{-4} at 16 Hz to less than 1×10^{-5} at 0.5 Hz (Hughes & Roth, 1974). It may be significant that in the nictitating membrane morphine has its most prominent effect at low frequencies of stimulation (0.1-0.4 Hz) at which the noradrenaline output is high in comparison to morphine-insensitive tissues. A similarly high noradrenaline output at low frequencies has also been observed in the morphine-sensitive mouse vas deferens (Henderson *et al.*, 1972a; Henderson & Hughes, unpublished observations).

In the cholinergic system, Greenberg, Kosterlitz & Waterfield (1970) have shown that at low stimulus frequencies acetylcholine release is higher from the guinea-pig myenteric plexus than from that of the rabbit; morphine inhibits acetylcholine release in the guinea-pig but not in the rabbit.

Therefore, in both the adrenergic and the cholinergic systems, the relationship between frequency and output at synapses at which morphine inhibits transmitter release is different from that at synapses at which morphine has no effect. The mechanisms underlying these observations are as yet unknown.

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INHIBITORY EFFECTS OF CLONIDINE AND BS 100-141 ON RESPONSES TO SYMPATHETIC NERVE STIMULATION IN CATS AND RABBITS

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1 In pithed cats, the spinal sympathetic outflow was stimulated preganglionically at segments C7 and T1 and heart rate responses and nictitating membrane tone were measured in parallel.

2 Clonidine and a related drug, BS 100-141 (N-amidino-2-(2,6-dichlorophenyl)acetamide hydrochloride), caused a dose-dependent inhibition of the stimulation-induced tachycardia but did not inhibit responses of the nictitating membrane. The inhibition of heart rate was antagonized by the α-adrenoceptor blocking drug, phentolamine.

3 In isolated hearts of rabbits, noradrenaline release in response to adrenergic nerve stimulation was reduced by clonidine and BS 100-141 and the effect was antagonized by phentolamine.

4 The results support the view that presynaptic α-adrenoceptors are involved in the regulation of transmitter release from adrenergic nerves. Cardiac adrenergic nerves appear more sensitive to α-adrenoceptor-mediated inhibition of impulse transmission than the sympathetic nerves to the nictitating membrane.

Introduction

Clonidine inhibits the effect of sympathetic stimulation on the heart in anaesthetized cats (Kobinger, 1967), pithed rats (Armstrong & Boura, 1973) and isolated hearts of rabbits (Starke & Altmann, 1973). Starke & Altmann (1973) have suggested that this effect is probably due to stimulation of α-adrenoceptors on postganglionic sympathetic nerve endings, which modulate transmitter release. That post-ganglionic nerves are endowed with α-adrenoceptors, stimulation of which causes a decrease and blockade an increase of transmitter release, has been demonstrated by Kirpekar & Puig (1971).

In the present paper, we have further tested the hypothesis that clonidine has an action on presynaptic α-adrenoceptors and have carried out comparative studies using a compound with similar effects, namely, N-amidino-2-(2,6-dichlorophenyl)-acetamide hydrochloride (BS 100-141) (Figure 1). The spinal sympathetic outflow in pithed cats was electrically stimulated and the inhibitory effects of these compounds on the heart rate and their antagonism by phentolamine were investigated. In other experiments the effects of these compounds on the output of noradrenaline in sympathetically stimulated rabbit hearts were measured. In the cat experiments, effects on the responses of the nictitating membrane to sympathetic nerve stimulation were examined.

Methods

Cats of either sex, 2-3 kg, were anaesthetized with pentobarbitone sodium (40 mg/kg i.p.) and pithed (Gillespie, Maclaren & Pollock, 1970) after bilateral cervical vagotomy. Artificial respiration was applied and an intravenous infusion of

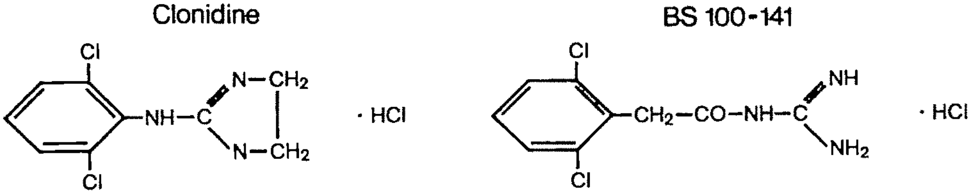

Figure 1

suxamethonium, 50 μg kg^{-1} min^{-1}, was given during the whole experiment. Body temperature was maintained at 37° C. Femoral arterial blood pressure was measured by means of a Statham P23 Db transducer. Integrated heart rate was recorded by a cardiotachometer. The left nictitating membrane was connected to a strain gauge transducer and pre-loaded with approximately 2 grams. Blood pressure, heart rate and isometric contractions of the nictitating membrane were recorded by means of an Offner S-II polygraph.

A pithing rod was used for stimulation of the spinal sympathetic outflow. The rod consisted of three sections: the cranial part (29 cm) and the caudal part (20 cm), both made of Perspex and connected to a short central section (2 cm) made of stainless steel. The rod had a uniform outer diameter of 4 mm. The middle part, used as the stimulation electrode, was placed, under X-ray monitoring, close to the spinal segments C7 and T1. The indifferent electrode was placed subcutaneously on the back.

The following stimulation procedure was adopted. Trains of supramaximal shocks (50 V) of 1 ms duration were applied for periods of 5 or 10 seconds. Each train of stimuli was followed by a 5 min interval, after which another stimulus train at the next higher frequency was applied. The initial stimulation frequency was usually 1 Hz and this was successively doubled until a frequency of 8 Hz was reached. Stimulation caused a transient increase in the heart rate which was followed by complete recovery to the original base line during intervals. The increase in heart rate was graded according to the stimulation frequency as shown in Figure 2. In 10 control experiments, stimulation with 0.5, 1, 2, 4, 8 and 16 Hz produced mean increases in heart rate of 5, 13, 28, 44, 58 and 67 beats/min respectively. Figure 2 also shows graded contractions of the nictitating membrane that depend on stimulation frequency.

Rabbit isolated hearts were perfused via the coronary vessels with Tyrode solution and their postganglionic sympathetic nerves stimulated (Huković & Muscholl, 1962). Two or three stimulation periods of 1 min duration (50 V, 3 ms, 7 Hz) were applied in each experiment. The time interval between successive stimulation periods was 15 minutes.

Perfusate collection lasted for 2 min from the onset of electrical stimulation. In the experiments of Table 1, drugs were infused from 10 min before the start of the second stimulation period. In those of Table 2, BS 100-141 was added 10 min before the start of the second stimulation period and subsequently left in the perfusate. Phentolamine was added 10 min before the start of the third stimulation period and subsequently left in the perfusate. Noradrenaline levels were determined fluorimetrically (Anton & Sayre, 1962).

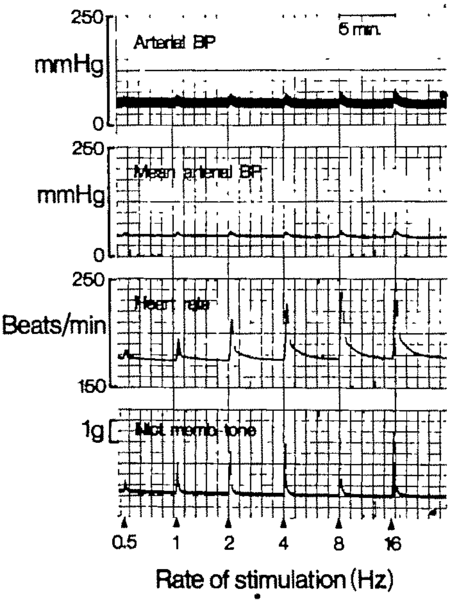

Figure 2 Stimulation of the spinal sympathetic outflow at segments C7 and T1 in a pithed cat. Stimulation parameters: 50 V, 1 ms during 10 s, with frequencies indicated at arrows. The tracings show effects on (from top) pulsatile femoral arterial blood pressure, mean blood pressure, heart rate and nictitating membrane tone (isometric recording).

Results

Heart rate in cats

Clonidine or BS 100-141 administered intravenously in doses of 5 and 15 μg/kg 5 min before beginning electrical stimulation of spinal segments C7 and T1 did not affect the resting rate but antagonized stimulation-induced increases in heart rate. As shown in Figure 3, the stimulation frequency-response curves were displaced to the right in a dose-dependent manner. The displacement occurred with both clonidine and BS 100-141 but was greater with the former drug. Within the range of stimulation frequencies employed, the stimulation frequency-response curves remained approximately parallel.

These effects of clonidine and BS 100-141 on the heart rate responses persisted for several hours but when the α-adrenoceptor blocking drug phentolamine (1 mg/kg) was administered 20 min after the higher dose of clonidine or BS 100-141,

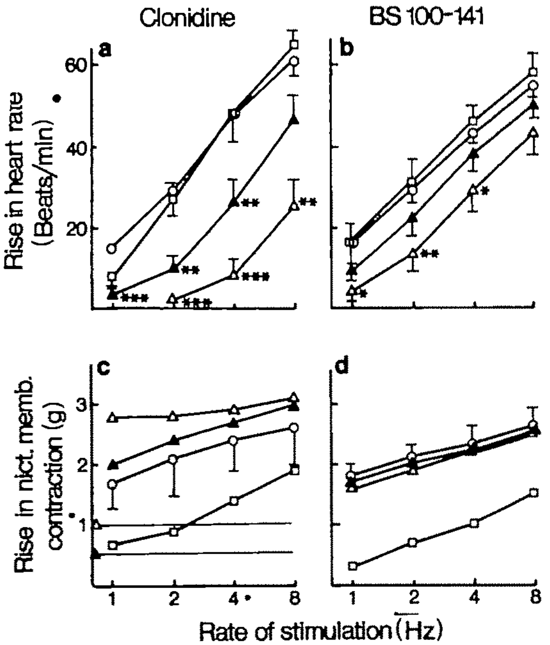

Figure 3 Responses of heart rate (a, b) and nictitating membrane (c, d) to stimulation of the spinal sympathetic outflow in pithed cats before (○) and after 5 μg/kg (▲) and 15 μg/kg i.v. (△) of clonidine or BS 100-141 and after a subsequent injection of phentolamine (1 mg/kg i.v.) (□). The time interval between drug injections was 20 minutes. The horizontal lines at the bottom of section (c) indicate the rise of basic tone in the nictitating membrane obtained with clonidine 5 μg/kg (▲) and 15 μg/kg i.v. (△) in the absence of nerve stimulation. Values are the mean for five cats. Vertical lines show s.e. mean. Significant difference from control: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (Student's *t* test).

their effects on heart rate were completely abolished (Figure 3a, b).

Contraction of the nictitating membrane

BS 100-141 had no appreciable contractile effect of its own and did not antagonize significantly the contractile effects of electrical stimulation of the nictitating membrane (Figure 3d) at doses which clearly antagonized sympathetic stimulation of the heart rate. Clonidine had a definite contractile effect of its own on the nictitating membrane so that the contractile effects of sympathetic stimulation were superimposed on an already raised base line (Figure 3c). Sympathetic stimulation in the presence of clonidine produced larger contractions than in its absence and although the results are not strictly comparable owing to the differences in base line there was no evidence that clonidine antagonized the effects of sympathetic stimulation on the nictitating membrane as it did in the heart.

As expected from an α-adrenoceptor blocking agent, phentolamine antagonized stimulation responses of the nictitating membrane (Figure 3c, d).

Noradrenaline output in rabbit hearts

In rabbit isolated hearts the release of noradrenaline following sympathetic nerve stimulation was significantly reduced by clonidine and BS 100-141 (Table. 1). In a further set of experiments (Table 2), phentolamine as well as BS 100-141 was used. BS 100-141 was perfused during the second and third stimulation periods and phentolamine during the third stimulation period. BS 100-141 inhibited noradrenaline output and phentolamine reversed the inhibition.

Table 1 Effects of BS 100-141 and clonidine on the output of noradrenaline (NA) during sympathetic nerve stimulation in rabbit isolated hearts

Drug	*Drug concentration (μg/ml during S_2)*	*NA output (ng per stimulation period)* S_1	S_2	n
Control	0	53 ± 6	51 ± 9	20
BS 100-141	0.04	41 ± 8	22 ± 5*	4
	0.4	42 ± 7	16 ± 3**	4
Clonidine	0.004	66 ± 10	42 ± 13*	8
	0.04	59 ± 9	34 ± 5**	6
	0.4	43 ± 8	15 ± 3**	6

The outputs S_1 and S_2 are prior to and in the presence of drug respectively. Mean values ± s.e. mean. *n* = number of experiments.

* $P < 0.05$; ** $P < 0.01$; *t* values were calculated by comparing S_1–S_2 in controls and after drug administration

Table 2 Effects of BS 100-141 (0.04 μg/ml) and phentolamine (0.12 μg/ml) on noradrenaline (NA) output during sympathetic nerve stimulation in rabbit isolated hearts

NA output (ng per stimulation period)		
S_1	S_2	S_3
Control	*BS 100-141*	
53 ± 7	22 ± 4	16 ± 4
		+ Phentolamine
59 ± 7	29 ± 4	69 ± 18**

Means of five determinations ± s.e. mean. S_1, S_2 and S_3 are successive stimulation periods.
** $P < 0.01$ for values of S_3-S_2 with and without phentolamine.

Discussion

The experiments show that the effect of clonidine on the heart has a peripheral component in addition to its known central component (Schmitt, Schmitt & Fenard, 1971). Similar peripheral effects were shown by both clonidine and BS 100-141 and they can be explained by assuming that these drugs stimulate presynaptic α-adrenoceptors that inhibit transmitter release as postulated by Kirpekar & Puig (1971). In favour of this explanation is the finding that both drugs produced a dose-dependent inhibition of sympathetic cardioacceleration which could be antagonized by phentolamine. The experiments on the sympathetically stimulated rabbit heart provided direct evidence of inhibition of noradrenaline release by clonidine confirming Starke & Altmann (1973). Our experiments show that BS 100-141 also inhibits stimulation-induced noradrenaline release and that this effect is antagonized by phentolamine.

Inhibitory effects of clonidine on sympathetic cardioacceleration had previously been demonstrated by Kobinger (1967) and Armstrong & Boura (1973), both finding that the inhibitory effects occurred preferentially at low stimulation frequencies. Our own experiments showed no evidence of preferential inhibition between the frequencies of 1 to 8 Hz. Possibly the discrepancy is due to differences in stimulation procedure, such as the very short stimulation periods used in the present work.

Our results indicate a certain cardioselectivity of clonidine-like α-adrenoceptor stimulant drugs. Doses of clonidine and BS 100-141 which produced a marked inhibition of the effect of stimulation of the sympathetic outflow to the heart did not influence the maximum tension developed in the nictitating membrane during stimulation of its sympathetic outflow. Guanabenz shows similar effects (Scholtysik, 1974). The cause of this cardioselective adrenergic neurone inhibition remains unknown.

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THE EFFECT OF PROPRANOLOL ON VASCULAR RESPONSES TO SYMPATHETIC NERVE STIMULATION

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1 In an attempt to clarify the rôle of the sympathetic neurone in the antihypertensive action of propranolol, the effect of this drug on responses to lumbar sympathetic nerve stimulation has been studied in the perfused hind-limb of the dog.

2 No consistent reduction of maximal or submaximal responses to nerve stimulation was produced by propranolol (10 to 100 μg/kg). In contrast, potentiation of nerve-evoked responses, as well as those to injected noradrenaline, usually occurred. Dexpropranolol (50 μg/kg) had no effect.

3 When neuronal uptake of noradrenaline was inhibited by desmethylimipramine or cocaine, no reduction in responses to sympathetic nerve stimulation was observed with propranolol.

4 No evidence was found, using α-adrenoceptor blocking drugs, that released transmitter stimulates β-adrenoceptors in the blood vessels of the hind-limb.

5 No evidence has been found for the existence of an adrenergic neurone-blocking action of propranolol that might contribute to the antihypertensive activity in man.

Introduction

Several hypotheses have been advanced for the antihypertensive action of propranolol. Prichard & Gillam (1969) postulated that the hypotensive effect resulted from a resetting of the baroreceptors, caused by reduction of transient increases in blood pressure of cardiac origin, which thereby regulate the blood pressure at a lower level. Other workers have suggested that a reduction in cardiac output is the primary cause of the antihypertensive effect (Frohlich, Tarazi, Dustan & Page, 1968; Dorph & Binder, 1969) or that decrease of renin release is important (Bühler, Laragh, Vaughan, Brunner, Gavras & Baer, 1973). Some *in vitro* studies have indicated that a pre-junctional block of sympathetic nerve activity may be produced by propranolol, although there is disagreement as to its mechanism and to its relevance *in vivo* (Day, Owen & Warren, 1968; Mylecharane & Raper, 1970; Barrett & Nunn, 1970). Recently, Eliash & Weinstock (1971) reported that propranolol in low doses reduced contractions of the nictitating membrane of cats by adrenergic neurone-blockade whereas high doses had no effect or caused potentiation. Raper & Wale (1969) also indicated that propranolol in high doses had no inhibitory effect in this preparation. Since responses of non-vascular tissue were employed in these findings, extrapolation to nerves innervating vessels may not be valid.

In addition to possible effects on nerves, propranolol also blocks vascular β-adrenoceptors, an action which could contribute to potentiation of sympathetic nerve-evoked pressor responses. However, β-receptors which mediate vasodilatation are believed not to be activated by noradrenaline released from nerves (Glick, Epstein, Wechsler & Braunwald, 1967; Brick, Hutchison & Roddie, 1967), although Viveros, Garlick & Renkin (1968) obtained activation of β-receptors during nerve stimulation.

We have investigated the effect of propranolol, in a range of doses, on vascular responses to sympathetic nerve stimulation and to injected noradrenaline in an attempt to determine whether sympathetic neurones may be involved in the antihypertensive action of propranolol.

Methods

Hind-limb perfusion experiments

Beagle dogs of either sex, weighing 6.5-13.0 kg, were anaesthetized with chloralose (120 mg/kg) injected intravenously and anaesthesia was maintained with chloralose (10 mg/kg) administered every 15 minutes. The left hind-limb was perfused at a constant rate with blood taken from a cannulated femoral artery, via a roller pump (Watson-Marlow, Model MHRE) and re-introduced distally into the same artery. The limb perfusion pressure was measured by means of a transducer (Bell & Howell, Model 4-327-L221) connected to a T-piece in the inflow tubing. The perfusion rate was adjusted so that the perfusion pressure, which reflects the vascular resistance, equalled the mean systemic arterial blood pressure. The paw circulation was occluded by tight ligatures.

The lumbar sympathetic chain was exposed at the level of L5, ligated and cut. The cut end was placed over bipolar platinum electrodes and stimulated with square-wave pulses of 1 ms duration at a frequency of 10 Hz. The stimulus strength was adjusted to give submaximal or maximal increases in resistance of the perfused limb.

The systemic arterial blood pressure was measured from the right femoral artery and heart rate recorded by means of a cardiotachometer (Horsfall, 1965) using subcutaneous electrodes. The right femoral vein was cannulated for injection of drugs. Drugs were also administered intra-arterially via a needle in the circuit immediately before the pump. Heparin, 1000 units/kg, was injected intravenously before perfusion was started.

Nictitating membrane preparation

Cats of either sex, weighing 1.4-2.1 kg, were anaesthetized with chloralose (80 mg/kg) injected intravenously either with or without induction of anaesthesia with ether. The left nictitating membrane was attached to a strain gauge (Devices Dynamometer UF1) by means of a silk thread. The left cervical sympathetic nerve was stimulated preganglionically via platinum electrodes with supramaximal shocks of 0.5 ms duration at 10 Hz for 15 s every 2 minutes. The isometric contractions of the nictitating membrane were recorded on a Devices M19 recorder. Blood pressure and heart rate were recorded as above.

Drugs

The following drugs were used: atropine sulphate (BDH), cocaine hydrochloride (BDH), desmethylimipramine (Geigy), dibozane [(1,4-benzodioxan-2-yl-methyl) piperazine] (McN-181, McNeil Laboratories), ergotamine tartrate (Sandoz), guanethidine sulphate (Ciba), isoprenaline sulphate (BDH), noradrenaline bitartrate (Burroughs Wellcome), phenoxybenzamine hydrochloride (Smith, Kline and French), propranolol hydrochloride (Inderal, ICI) and dexpropranolol (ICI). In each experiment, atropine sulphate (1 mg/kg) was injected intravenously every hour. All doses are expressed in terms of the salt with the exception of dexpropranolol.

Experiment protocol

Control responses of the hind-limb perfusion pressure to nerve stimulation, isoprenaline and noradrenaline were obtained and the mean for each calculated. Propranolol was then injected intravenously and nerve stimulation and drug injection repeated. The means of responses obtained during a 30 min period after administration of propranolol were compared with the control means, being expressed as percentage change, and the significance of differences determined by Student's *t* test. For a given dose of propranolol used in at least three different experiments, the mean changes in responses were grouped and the mean obtained.

In the experiments in which α-adrenoceptor blockade was investigated, the blocking drugs were administered intravenously or intra-arterially to the anaesthetized dogs, in the case of phenoxybenzamine the drug was also given to the animal on one or two days before the experiment.

Results

Effects of propranolol

Typical responses to lumbar sympathetic nerve stimulation, and to intravenously injected noradrenaline and isoprenaline on hind-limb resistance, blood pressure and heart rate are shown in Figure 1. The effect of propranolol in one experiment is shown graphically in Figure 2 and the results from all experiments with propranolol (10-100 μg/kg i.v.) are given in Table 1. In only one experiment was there a significant reduction in the maximal nerve-evoked response of the hind-limb resistance following any of the doses of propranolol used. Indeed, a potentiation of maximal responses was usually seen.

The responses to submaximal stimulation were also potentiated except in one experiment (the same one in which the maximal response was reduced) when propranolol 50 μg/kg reduced these responses by 60%. In general, the size of the potentiation was greater with the higher doses of propranolol. The responses to injected noradrenaline were also increased by propranolol which, even in the lowest doses, produced a degree of β-adrenoceptor blockade as judged by the responses to intravenously injected isoprenaline (see Figure 2 and Table 1).

An intravenous dose of guanethidine 5 mg/kg

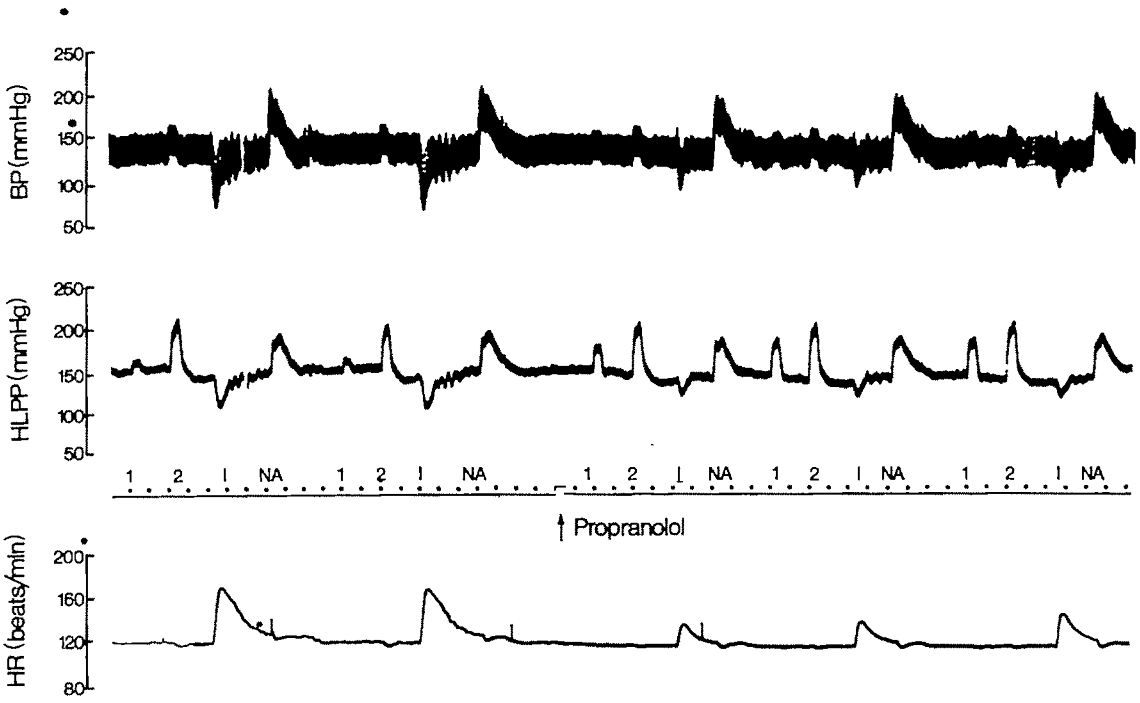

Figure 1 Effect of propranolol (50 μg/kg i.v.) on responses of the blood pressure (BP), hind-limb perfusion pressure (HLPP) and heart rate (HR) to submaximal (1) and supramaximal (2) lumbar sympathetic stimulation, isoprenaline (I, 0.2 μg/kg i.v.) and noradrenaline (NA, 0.2 μg/kg i.v.) in the dog. Time scale: min.

abolished constrictor responses to lumbar sympathetic nerve stimulation within 1 hour. Responses to noradrenaline were enhanced, whilst those to isoprenaline were unchanged.

Inhibition of noradrenaline uptake

It is possible to potentiate responses to sympathetic nerve stimulation and injected noradrenaline by inhibiting the uptake of noradrenaline into neuronal and other sites. This inhibitory effect, leading to increased sympathetic responses, could mask possible neuronal blocking actions of propranolol. Experiments were therefore carried out following the maximal potentiation of responses to nerve-released and injected noradrenaline by desmethylimipramine and cocaine, drugs known to inhibit uptake of transmitter. Pretreatment with these drugs did not modify the enhancement by propranolol of nerve-induced

Table 1 Effect of propranolol, injected intravenously, on responses of the vascular resistance of the perfused dog hind-limb to supramaximal and submaximal lumbar sympathetic stimulation, noradrenaline (0.2 μg/kg i.v.) and isoprenaline (0.2 μg/kg i.v.)

Propranolol (μg/kg)	*No. of expts*	*Effect on responses to:*			
		Submax. stimulation	*Supramax. stimulation*	*Noradrenaline*	*Isoprenaline*
		(Percentage change ± s.e. from mean control responses)			
10	5	+33 ± 11*	+14 ± 5*	+29 ± 9*	−27 ± 7**
25	3	+61 ± 30	+26 ± 10	+66 ± 8	−54 ± 2*
50	5	+76 ± 23*	+27 ± 7*	+31 ± 23	−58 ± 4***
	1	−39	−61	−31	−63
100	4	+97 ± 30*	+23 ± 13	+29 ± 17	−83 ± 3***

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

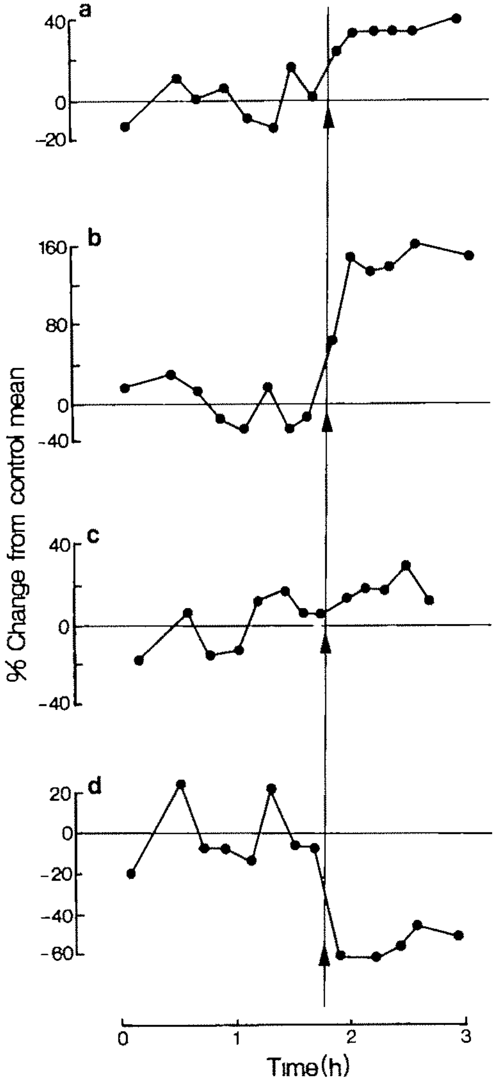

Figure 2 Effect of propranolol, 50 μg/kg i.v. (injected at arrow) on responses of the dog hind-limb perfusion pressure to (a) supramaximal, (b) submaximal lumbar sympathetic stimulation, (c) noradrenaline (0.2 μg/kg i.v.) and (d) isoprenaline (0.2 μg/kg i.v.).

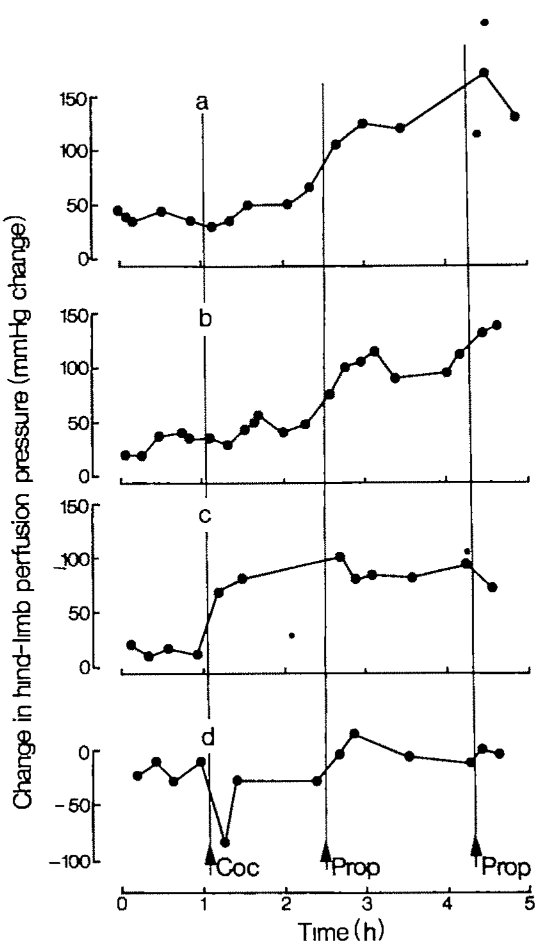

Figure 3 Changes in dog hind-limb perfusion pressure in response to (a) supramaximal and (b) submaximal lumbar sympathetic stimulation, (c) noradrenaline (0.2 μg/kg i.v.) and (d) isoprenaline (0.2 μg/kg i.v.). At the first arrow (Coc), cocaine (3 mg/kg i.v.) was administered and at the second and third arrows (Prop), propranolol (50 μg/kg i.v.) was given.

responses, although no potentiation of responses to injected noradrenaline occurred after propranolol. The hind-limb perfusion pressure increased for about 15 min following the administration of cocaine and then returned to the control base-line where it remained for the duration of the experiment. The resting heart rate did not change following cocaine. Figure 3 shows the effect of propranolol following an intravenous injection of cocaine 3 mg/kg.

Blockade of α-adrenoceptors

If released transmitter activated β-adrenoceptors, which mediate vasodilatation in the skeletal muscle bed, blockade of these receptors would potentiate the constrictor effect. Attempts were therefore made to abolish, by means of α-receptor blocking drugs, the constrictor action of released noradrenaline. If β-receptor activation were involved in the action of the transmitter, a dilatation should occur on nerve stimulation.

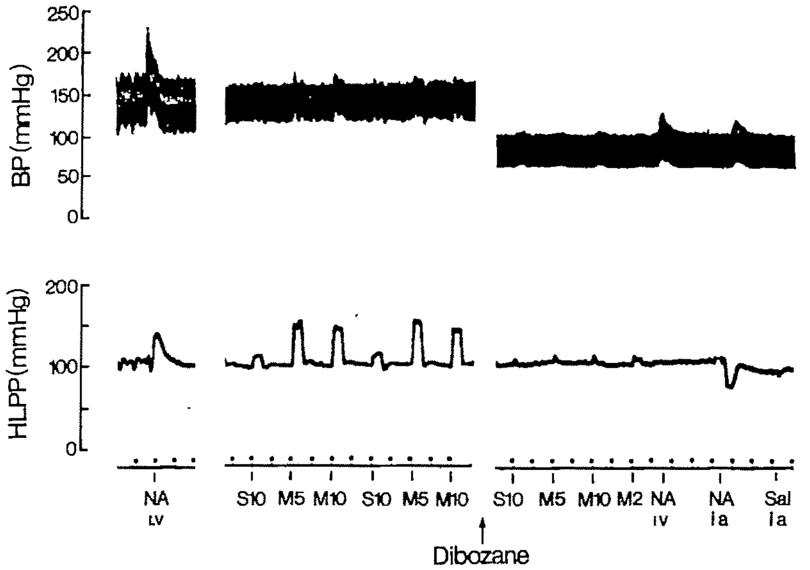

Figure 4 Effect of dibozane (400 μg/min i.a.) on responses of the blood pressure (BP) and hind-limb perfusion pressure (HLPP) to lumbar sympathetic stimulation and noradrenaline (NA 0.5 μg/kg). The nerve was stimulated at submaximal (S) or maximal (M) strength at the frequencies (Hz) indicated by the numbers. Noradrenaline was injected intravenously (i.v.) or intra-arterially (i.a.). An injection of 0.9% w/v NaCl solution (Sal) was also given intra-arterially. The final section of the record was obtained 80 min after starting the infusion of dibozane. Time scale: min.

Phenoxybenzamine (10-20 mg/kg i.v.) reduced the constrictor effect of nerve stimulation but never abolished it. Even by pretreating the animal on three days preceding the experiment (10 mg/kg i.v. per day), small constrictor responses were still evident on stimulation of the lumbar sympathetic nerve. However, by using ergotamine (50 μg/kg i.a. and 200 μg/kg i.v.) in addition to phenoxybenzamine, constrictor responses were abolished, but no dilatation was seen during nerve stimulation.

Using the α-receptor blocker, dibozane (infusion of 400 μg/min i.a.), the constrictor responses were abolished but again no dilatation occurred in response to nerve stimulation (Figure 4), although noradrenaline (0.5 μg/kg) administered intra-arterially into the perfused limb produced vasodilatation. In one experiment in which a combination of phenoxybenzamine and ergotamine were used to produce the α-receptor blockade, vasodilatation was observed on nerve stimulation. However, the responses were unaffected by propranolol (0.03-3 mg/kg i.v.).

Propranolol (0.2 mg/kg i.v.) had little effect on residual constrictor responses during α-receptor blockade but abolished the vasodilatation produced by intra-arterially injected noradrenaline.

Dexpropranolol

Dexpropranolol (50 μg/kg i.v.) had no effect on the responses to lumbar sympathetic nerve stimulation whereas the same dose of racemic propranolol in the same animal exerted the usual potentiating effect.

Experiments on the nictitating membrane of the cat

The experiments of Eliash & Weinstock (1971) were repeated as closely as possible, including induction of anaesthesia with ether before administering chloralose, using isometric contractions of the membrane and employing the same doses of propranolol. In no experiment did propranolol in intravenous doses of 50-150 μg/kg reduce contractions of the nictitating membrane. In five experiments, 50 μg/kg propranolol (the optimal dose for reduction of membrane contractions in the experiments of Eliash & Weinstock) was injected intravenously and the effects on contractions observed for up to 90 min (Figure 5). Although an isoprenaline-induced tachycardia was reduced by the propranolol, no reduction in membrane contractions was seen. Additional

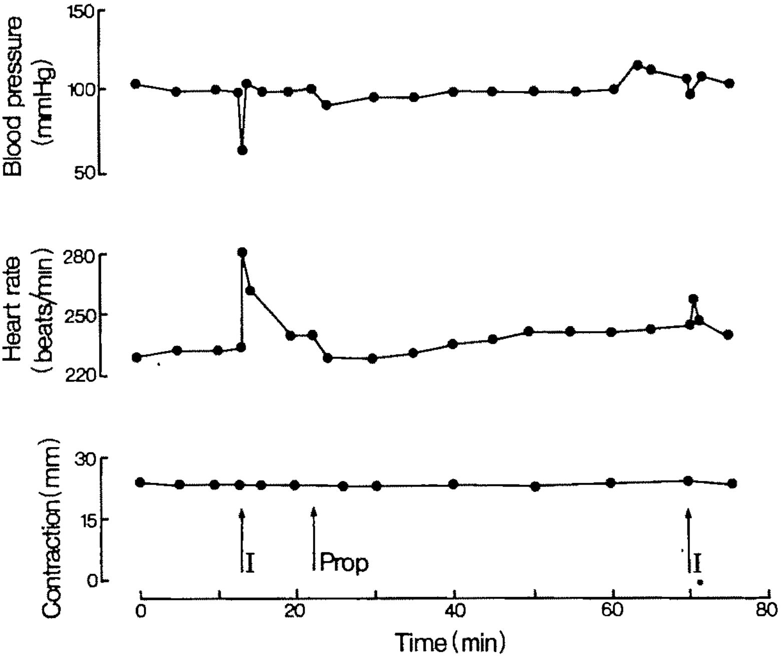

Figure 5 Effect of isoprenaline, 0.1 μg/kg (I) and propranolol, 50 μg/kg (Prop) administered intravenously on the blood pressure, heart rate and size of contractions of the nictitating membrane of an anaesthetized cat.

intravenous doses of 50 μg/kg also did not reduce the magnitude of the contractions. No reduction in blood pressure was produced by these doses of propranolol in our experiments and there was also no potentiation of the responses of the nictitating membrane.

Discussion

In these experiments in atropinized, anaesthetized dogs, using vascular responses elicited by stimulation of sympathetic nerves, no inhibitory effect was produced by propranolol over a 10-fold dose range. In contrast, potentiation was usually seen, even when uptake of released transmitter was inhibited by desmethylimipramine or cocaine. Similarly, Glick *et al.* (1967) and Levin & Beck (1967) observed no inhibition by propranolol of sympathetic constrictor responses and Raper & Wale (1968) did not report any inhibition of nerve-evoked contractions of the nictitating membrane. All these authors used a single dose level of propranolol, usually above 1 mg/kg. However, even with lower doses of propranolol (10 to 100 μg/kg), we have been unable to reproduce the neurone blockade reported by Eliash & Weinstock (1971). These latter authors used the nictitating membrane preparation and extrapolated their findings to resistance vessels in order to explain the antihypertensive action of propranolol. Although various sympathetic nerves might be expected to react in the same manner to neurone-blocking drugs, differences in the end-organ may affect the results. For example, it has been reported that the nictitating membrane is devoid of β-receptors (see Levin & Beck, 1967), or has only weakly reactive ones (Smith, 1963), whereas arteries and arterioles are known to respond readily to β-receptor stimulation. However, even in the nictitating membrane preparation we have been unable to detect a depressant effect of propranolol using nerve stimulation. The reason for a discrepancy between our results and those of Eliash & Weinstock (1971) is not clear. Furthermore, in the experiments reported here, no fall in blood pressure is seen with these low doses of propranolol whereas Eliash & Weinstock (1971) show a marked fall.

Another factor which may influence the results obtained on blood vessels is inhibition of uptake of released noradrenaline. Propranolol in this respect is one tenth as potent as cocaine (Foo, Jowett & Stafford, 1968), and if a preparation were particularly sensitive to block of uptake, then a potentiation to released and injected noradrenaline might mask inhibitory effects. However, even following treatment with desmethylimipramine or cocaine, no inhibitory effect of propranolol on constrictor responses to sympathetic nerve stimulation was observed. It is possible that the doses of the uptake inhibitors did not completely block re-uptake of nerve-released transmitter and propranolol produced further potentiation by a cocaine-like action. There is no evidence that propranolol can enhance the release of noradrenaline from sympathetic nerves (Starke & Schumann, 1972).

Potentiation of the responses to injected noradrenaline by low doses of propranolol (10-100 μg/kg) could be due to blockade of the β-adrenoceptors which mediate vasodilatation. However, no evidence could be found in these experiments for activation of β-receptors by nerve-released noradrenaline. This is in agreement with the findings of Glick *et al.* (1967) and Brick *et al.* (1967), although Viveros *et al.* (1968) obtained a vasodilatation on nerve stimulation during α-receptor blockade with dibozane.

Eliash & Weinstock (1971) found that higher doses of propranolol (0.5 mg/kg) reversed the adrenergic neurone blockade produced by lower doses (25-100 μg/kg) in the cat and proposed that release of catecholamine from the adrenal medulla was responsible for this effect (Eliash & Weinstock, 1972a, b). However, we saw no change in perfusion pressure that might be indicative of an increased circulating level of catecholamine. There is, therefore, no evidence that adrenal stimulation is more apparent in the dog than in the cat.

Dexpropranolol, which has less than 1% of the potency of propranolol as a β-receptor blocking agent (Howe & Shanks, 1966), did not potentiate or reduce the responses to nerve stimulation. The fact that dexpropranolol is as potent as propranolol in blocking uptake of noradrenaline (Foo *et al.*, 1968), is further evidence that this effect is not responsible for the potentiation by propranolol of nerve-evoked constrictor responses. An effect due to a β-receptor blocking action seems likely, therefore, although evidence of a post-synaptic action on β-receptors has not been obtained.

If these doses of propranolol released adrenal catecholamines (Regoli, 1970) which stimulated the release of renin and thereby angiotensin, an increased noradrenaline release due to angiotensin (Zimmerman, 1962) could be responsible for the potentiation of nerve-evoked responses seen with propranolol. However, significant adrenal catecholamine release seems unlikely with these low doses of propranolol and this β-blocking drug is also known to reduce catecholamine-induced release of renin (Winer, Choksi & Walkenhorst, 1970; Assaykeen, Clayton, Goldfein & Ganong, 1970). Eliash & Weinstock (1971) showed that dexpropranolol also reduced contractions of the nictitating membrane although this drug does not exert an antihypertensive effect in man (Waal-Manning, 1970; Prichard, 1971).

No evidence has therefore been found to support the contention that a guanethidine-like neurone-blocking action of propranolol in low doses might contribute to its antihypertensive effect. Clinically, the antihypertensive dose of propranolol is usually larger than the minimum dose to block β-receptors whereas larger doses were found by Eliash & Weinstock (1971) to reverse the neurone blockade. Moreover, the guanethidine-like side-effects of postural hypotension and failure of ejaculation do not occur with propranolol.

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AN INDIRECT SYMPATHOMIMETIC EFFECT OF BURIMAMIDE ON KITTEN ISOLATED ATRIA

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1 Burimamide (34-1080 μM) caused a concentration-dependent increase in the force and frequency of contraction of kitten isolated atria.

2 Metiamide (467 μM) had no stimulant action on kitten atria and did not modify the effects of burimamide.

3 The atrial stimulation produced by burimamide was reduced by (–)-propranolol (34-68 nM) and by cocaine (3 μM)

4 The atrial stimulant effect of burimamide was prevented by pretreatment of kittens with reserpine (1 mg/kg, 24 h before the experiment).

5 It is concluded that burimamide causes atrial stimulation by releasing catecholamines

Introduction

Burimamide antagonizes the increase in gastric acid secretion induced by histamine, or pentagastrin, by blocking histamine H_2-receptors (Black, Duncan, Durant, Ganellin & Parsons, 1972). In addition, burimamide also stimulates acid secretion when injected into cats, possibly by acting as a partial agonist on the histamine H_2-receptors in the stomach (Harris, Reed, Smy & Venables, 1975). In the present study, the effects of burimamide on kitten isolated atria have been investigated in an attempt to determine whether burimamide also stimulates other histamine H_2-receptors in the cat. It has been found that burimamide possesses an appreciable cardiac stimulant action; this appears to be brought about by release of catecholamines, an effect previously reported in anaesthetized cats (Albinus & Sewing, 1973).

Methods

Kittens of either sex and weighing 400-900 g were anaesthetized with ether. Heparin (2500 u) was injected into a femoral vein. The heart was removed, washed in McEwen's solution (McEwen, 1956) at room temperature, and then transferred to the medium at 37° C. The atria were dissected from the ventricles and the preparation was placed in a 70 ml isolated organ bath. The McEwen's solution was maintained at 37 ± 0.5° C and was bubbled with 95% O_2 and 5% CO_2.

Contractions of the atria were monitored with an Ether ST202 force displacement transducer connected to a Devices M2 recorder. Heart rate was recorded with a Devices Impulse Integrator (type 3210) which was triggered from the transducer signal The atria were placed under an initial tension of 0.5-1.2 grams. Experiments were started after a 60 min equilibration period, during which time the bath fluid was changed six times.

Two types of experiment were performed: (a) concentration-response curve experiments, and (b) bracketing experiments consisting of single submaximal responses to noradrenaline, histamine, tyramine and burimamide. Concentration-response curves were constructed by giving an ascending series of doses, followed by washing out of each dose, this being continued until there were two or more identical increases in heart rate. The dosing procedure was as follows: noradrenaline, 3 min contact with the tissue on a 10 min cycle followed by six changes of bathing solution: histamine, 5 min contact on a 15 min cycle with 12 changes of bathing solution: burimamide, 5 min contact on a 20 min cycle with 18 changes of bathing solution. Each preparation in this series of experiments provided curves for all three agonist drugs.

In the bracketing experiments, submaximal responses of the atria to single doses of the agonists were matched. The three agonist drugs were then repeated in the presence of the modifying drug, propranolol and metiamide being added 1 min before each dose of agonist, whereas cocaine was allowed to act for 5 minutes.

Two kittens were pretreated with reserpine (1 mg/kg, i.p., 24 h before the experiment), the drug solution being prepared by the method of Burack, Weiner & Hagen (1960).

Results were calculated as the mean ± s.e. mean and analysed for significance of difference of means by Student's t test following an analysis of variance.

Drugs

The following drugs were used: tyramine hydrochloride and (–)-noradrenaline bitartrate (Koch-Light); histamine acid phosphate and reserpine (BDH); (–)-propranolol hydrochloride (ICI); cocaine hydrochloride (Mawson & Proctor); heparin (Evans); anaesthetic ether B.P. (May & Baker); burimamide and metiamide (S.K. & F.). Solutions of noradrenaline were protected from the light. Burimamide and metiamide were dissolved in the minimum volume of 10 N HCl, diluted with water, and taken to pH 7 with dilute NaOH.

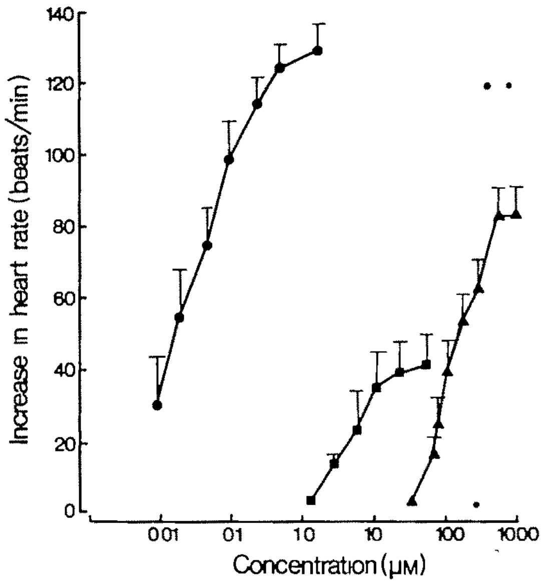

Figure 1 Concentration-response curves for the positive chronotropic response of kitten isolated atria to noradrenaline (●), histamine (■) and burimamide (▲). Vertical bars show s.e. mean of five experiments. Note that the maximum effect of burimamide was greater than that of histamine.

Results

Chronotropic and inotropic actions of burimamide

In 22 experiments the control heart rate was 157 ± 5 beats/min and the tension developed each beat was 760 ± 55 mg. Burimamide (34-1080 µM) stimulated the atria, causing both chronotropic and inotropic effects. There was no tachyphylaxis to the cardiac effects of 270 µM burimamide over the first three applications ($n = 5$), although there was some loss of response after the seventh application of this dose in one preparation.

The cardiac stimulant effects of burimamide have been compared with those produced by noradrenaline and histamine. The positive chronotropic effects of the three drugs are shown in Figure 1. The maximum responses were: noradrenaline 130 (beats/min increase in heart rate), burimamide 84 and histamine 41. Burimamide and noradrenaline also exerted substantial positive inotropic effects, but histamine produced only a small negative inotropic response. However, the response of the atria to histamine was very variable; on some occasions a small positive inotropic effect was seen (see Figure 2 for example), and in some of the later experiments a positive chronotropic response was observed which was greater than the maximum in Figure 1.

Effects of various pretreatments on the cardiac response to burimamide

Metiamide Metiamide (467 µM), a more potent histamine H_2-receptor antagonist than burimamide (Black, Duncan, Emmett, Ganellin, Hesselbo, Parsons & Wyllie, 1973), produced a small positive inotropic effect (<200 mg increase in tension,

Table 1 The effects of drugs on the positive chronotropic effects of agonists on kitten atria

			Response as % control (s.e. mean)			
Drugs	*µM*	n	*Noradrenaline (17-170 nM)*	*Histamine (10-20 µM)*	*Tyramine (2 µM)*	*Burimamide (270 µM)*
Metiamide	467	5	90 (10)	20 (10)*		104 (9)
(–)-Propranolol	0.034-0.068	6	40 (22)*	105 (10)		12 (4)*
Cocaine	3	5	134 (5)		28 (4)*	24 (7)*

* Signifies a significantly lower tachycardia after the application of a modifying drug ($P < 0.05$).

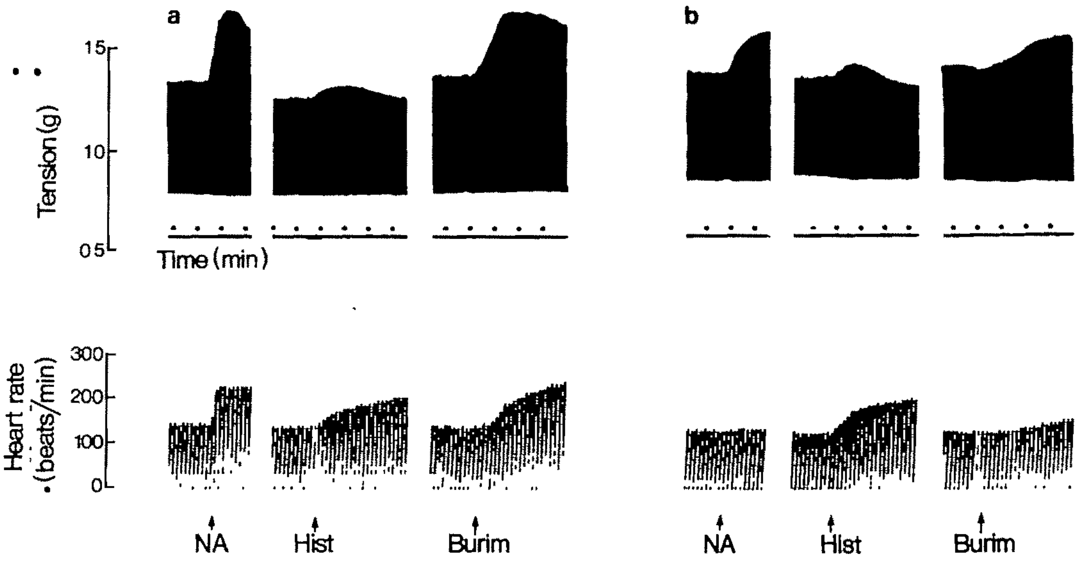

Figure 2 The effect of noradrenaline 85 nM (NA), histamine 10 μM (Hist) and burimamide 270 μM (Burim) on kitten isolated atria alone (a) and in the presence (b) of (—)-propranolol (68 nM added 1 min before each agonist). Note that the effects of noradrenaline and burimamide were reduced, but the sensitivity of the atria to histamine was unaffected.

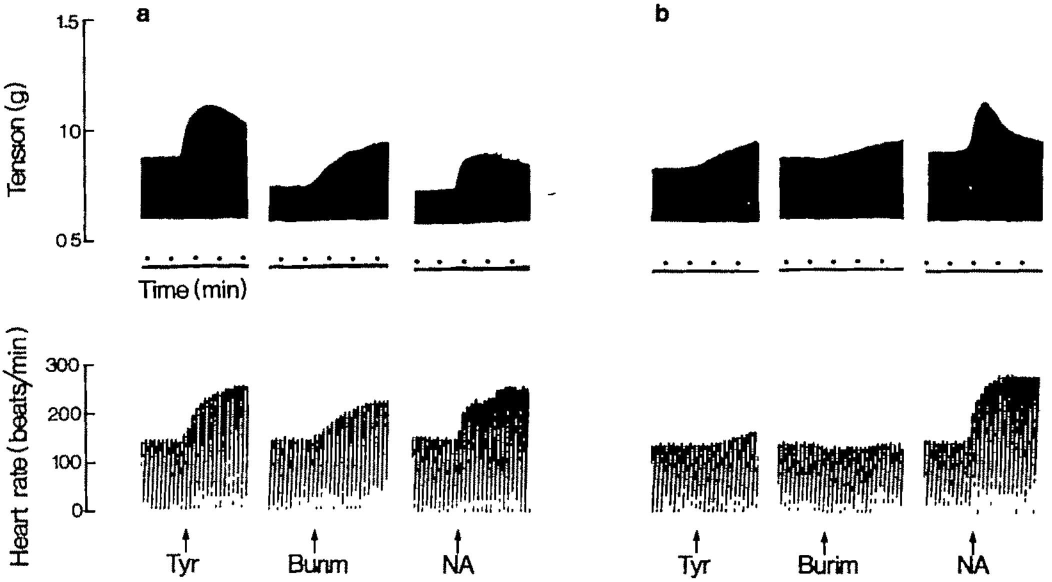

Figure 3 The effects of tyramine 2 μM (Tyr), burimamide 270 μM (Burim) and noradrenaline 42 nM (NA) before (a) and after (b) cocaine (3 μM added 5 min before each agonist). Note that the effects of tyramine and burimamide were reduced after cocaine whereas the effect of noradrenaline was increased.

Table 2 Effect of reserpine on the positive chronotropic effect of agonists on kitten atria

	Tachycardia (s.e. mean) beats/min		
	Noradrenaline (42 nM)	*Tyramine (2 µM)*	*Burimamide (270 µM)*
Control group	82 (12))	96 (8)	71 (4)
Reserpine-treated kitten 1	60	26	3
Reserpine-treated kitten 2	113	0	0

Reserpine (1 mg/kg, i.p.) was administered 24 h before the isolated atria were prepared. There were five preparations in the control group.

$n = 8$) which was usually preceded by a small negative chronotropic effect ($n = 6/8$). The subsequent application of the cardiac stimulants, noradrenaline (42-85 nM), histamine (10-20 µM) and burimamide (270 µM), revealed a specific antagonism of histamine (Table 1).

Propranolol and cocaine Sub-maximal responses to doses of noradrenaline, histamine and burimamide were tested before and then 1 min after (−)-propranolol (34-68 nM). Results of a typical experiment are shown in Figure 2. Propranolol reduced both the chronotropic and inotropic effects of noradrenaline and burimamide, but did not affect the sensitivity of the atria to histamine (see Table 1).

Cocaine (3 µM) reduced the effects of tyramine and burimamide, whereas noradrenaline was more effective than in the controls (Figure 3 and Table 1).

Reserpine Pretreatment of two kittens with reserpine (1 mg/kg, i.p., 24 h before the experiment) reduced the sensitivity of the atria to tyramine and prevented the stimulant action of burimamide (Table 2). Responses to noradrenaline were unaffected by reserpine.

Discussion

In the present experiments on the isolated, spontaneously-beating atria of the kitten there is no evidence to suggest that burimamide is a partial agonist on histamine H_2-receptors. The positive inotropic response to histamine of the atria was variable, and not always present; in contrast, burimamide consistently increased the frequency and force of contractions. The maximum effects of burimamide on both power and rate of beating were much greater than were those in the presence of histamine. The effects of burimamide were not blocked by metiamide, whereas those of histamine were antagonized. It can therefore be concluded that the cardiac stimulant action of burimamide in these experiments was not due to an effect on histamine H_2-receptors.

(−)-Propranolol significantly reduced the response to burimamide and noradrenaline without affecting sensitivity to histamine. It is unlikely that the 'non-specific' activity of propranolol (Papp & Vaughan Williams, 1969) contributed to the blockade of the effects of burimamide because of the low concentrations (34-68 nM) of the β-adrenoceptor blocking agent that were used. However, it was surprising that propranolol was more effective against burimamide than noradrenaline.

Experiments with cocaine and reserpine showed that the sympathomimetic effect of burimamide was an indirect effect, presumably caused by a release of noradrenaline from adrenergic neurones in the atria. Cocaine inhibits the active transport of monoamines into the neurone, thus preventing tyramine reaching the noradrenaline stores and by the same action increasing the concentration of exogenous noradrenaline in the vicinity of the receptors on the cardiac cells (MacMillan, 1959; Iversen, 1965). Since, in the present experiments, cocaine reduced the response of the atria to burimamide in a similar fashion to its effect on tyramine, it appears that burimamide can release catecholamines from the nerve endings by a tyramine-like action. This, considered with the insensitivity of the atria from the reserpine-treated kittens to both burimamide and tyramine, suggests that burimamide is an indirectly-acting sympathomimetic agent in this species.

Albinus & Sewing (1973) found that burimamide (4 mg/kg) increased blood pressure and heart rate in anaesthetized cats, and that these effects were antagonized by a mixture of α- and β-adrenoceptor blocking agents. The rise in blood pressure could also be prevented by acute adrenalectomy, but the tachycardia was not reduced. It is possible that a release of catecholamines in the heart, such as described in this paper, was responsible for the tachycardia which occurred in their experiments.

Cardiovascular effects following burimamide have also been seen in dogs (Lorenz, Thermann, Hamelmann, Schmal, Maroske, Reimann, Kusche, Schingale, Dormann & Keck, 1973). In man, Wyllie, Hesselbo & Black (1972) observed an increase in heart rate from 81 beats/min to 96 beats/min during infusion of burimamide. The plasma concentration of burimamide in the experiments in man reached a maximum of 40 μM, a concentration that produced a tachycardia in the kitten isolated atria. However, although there are no data on the plasma concentration of burimamide accompanying histamine H_2-receptor blockade in the kitten, it is probable that appreciable indirect sympathomimetic activity occurs only at concentrations of burimamide greater than those needed for receptor blockade. In contrast with the present findings for burimamide, there was no evidence that metiamide released catecholamines from the heart. This represents yet another advantage of metiamide when compared with burimamide.

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EFFECT OF ACETYLCHOLINE ON CHANGES IN CONTRACTILITY, HEART RATE AND PHOSPHORYLASE ACTIVITY PRODUCED BY ISOPRENALINE, SALBUTAMOL AND AMINOPHYLLINE IN THE PERFUSED GUINEA-PIG HEART

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1 Isolated perfused hearts of guinea-pigs were given graded doses of isoprenaline, salbutamol and aminophylline, both before and during acetylcholine infusion.

2 The three agonists produced increases in contractile force, heart rate and ventricular glycogen phosphorylase activity.

3 Acetylcholine, in the concentration used, had no effect on any of the measured variables but did antagonize the effects of the three agonists on contractility and phosphorylase activity. The positive chronotropic responses were unaltered by acetylcholine infusion.

4 The ratio of the dose required for a standard heart rate response to the dose producing a standard contractile force response was different for each agonist.

5 The selective antagonism of the contractile response to isoprenaline, salbutamol and aminophylline suggest that different mechanisms are involved in the initiation of positive inotropic and chronotropic responses.

Introduction

Cardiac β-adrenoceptor stimulation produces increases in both heart rate and contractile force. It has been proposed that stimulation of cardiac β-adrenoceptors by catecholamines leads to enhanced cyclic adenosine 3′,5′-monophosphate (cyclic AMP) production which acts as a 'second messenger' to initiate the positive inotropic and chronotropic responses (Robison, Butcher, Øye, Morgan & Sutherland, 1965; Kukovetz & Pöch, 1972). However, a low concentration of acetylcholine that does not affect ventricular contractility, rate, phosphorylase activity, or cyclic AMP formation has been shown to antagonize the initial adrenaline-induced increases in contractility, phosphorylase activity, and cyclic AMP formation without affecting the positive chronotropic effect (Chamales, Williams, Pauk & Ellis, 1971). These results have led to the proposal that cardiac inotropic and chronotropic responses may be mediated by different mechanisms. The purpose of the present investigation was to test this hypothesis by studying the cardiac responses to isoprenaline, aminophylline and salbutamol, both alone and in combination with acetylcholine. It has been suggested that isoprenaline and aminophylline produce their cardiac effects through acceleration of synthesis of cyclic AMP and inhibition of cyclic AMP degradation, respectively (Robison, Butcher & Sutherland, 1971). Salbutamol is considered to act selectively on β_2-adrenoceptors (Cullum, Farmer, Jack & Levy, 1969), but produces effects on the heart qualitatively similar to those of isoprenaline, a less selective β-adrenoceptor agonist. Acetylcholine has been shown to reduce the elevated cyclic AMP concentrations seen after addition of adrenaline or isoprenaline to particulate preparations from heart tissue (Lee, Kuo & Greengard, 1971; Kuo, Lee, Reyes, Walton, Donnell & Greengard, 1972) or after catecholamine administration to guinea-pig isolated hearts (Chamales *et al.*, 1971). It has been proposed that this effect at the cyclic AMP level may be the basis for the observed acetylcholine-adrenaline antagonism (LaRaia & Sonnenblick, 1971).

The present study examines the interactions between drugs thought to act by increasing cyclic AMP accumulation, and acetylcholine which acts by decreasing cyclic AMP concentration, to determine whether cyclic AMP accumulation is directly related to the production of both heart rate and contractile force responses.

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Methods

Isolated perfused hearts

Guinea-pigs of either sex exceeding 400 g in weight were injected with heparin, and their hearts quickly removed under pentobarbitone anaesthesia. Hearts were perfused at 27°C by the Langendorff technique using Krebs-Henseleit bicarbonate solution containing 0.2% glucose and equilibrated with 95% O_2 and 5% CO_2 (Vincent & Ellis, 1963). All hearts were perfused at a pressure of 40 cm of water and were allowed to stabilize for 30 min before any drug infusion. Contractile force was recorded on a Grass polygraph via a Grass FT-03 force displacement transducer attached to the apex of the ventricles. Heart rate was determined from the contractile force recording. The calibration of the recording system before each experiment was performed as described by Blukoo-Allotey, Vincent & Ellis (1969). Diastolic tension was adjusted to 10 g before drug administration was begun. All hearts used for phosphorylase activity determinations received only one injection. Hearts receiving multiple drug injections were allowed recovery periods sufficient to reestablish pre-injection heart rate values.

Acetylcholine iodide, stored as a 10 mg/ml stock solution at 4°C, and isoprenaline hydrochloride, aminophylline, and salbutamol, prepared daily as stock solutions, were further diluted with 0.9% w/v NaCl solution (saline) immediately before administration. Acetylcholine was administered by infusion delivered into the aortic cannula by a syringe pump at a rate of 0.1 ml/minute. Isoprenaline, aminophylline, or salbutamol was administered into the aortic cannula by bolus injection in volumes of 0.2 ml or less.

Increasing concentrations of isoprenaline, aminophylline or salbutamol were alternately injected alone and 2 min after starting an infusion of 0.018 μg/min of acetylcholine. Acetylcholine infusions were allowed to continue 5 min after each drug injection and then were turned off for at least 10 min before the next drug injection.

Hearts used for phosphorylase activity determinations were frozen 30 s or 1 min after drug injection by clamping between modified Wollenberger tongs (Wollenberger, Ristau & Schoffa, 1960) precooled in liquid nitrogen. Tissues were stored in liquid nitrogen until biochemical assays were performed. Phosphorylase activity was determined in the hearts used for physiological studies.

Biochemical methods

Tissue samples were excised from the apex region of the ventricle and weighed in a refrigerated room (−20° C). Phosphorylase activity was determined in both the presence and absence of adenosine 5′-monophosphate by the enzymatic method of Hardman *et al.* (1965). Phosphorylase activity is expressed as percent phosphorylase *a*

$$\left(\frac{\text{phosphorylase } a \text{ activity}}{\text{total phosphorylase activity}}\right) \times 100$$

Drugs and chemicals

Acetylcholine iodide and aminophylline were purchased from Sigma Chemical Co. Salbutamol was obtained from the Schering Corp., U.S.A. and (−)-isoprenaline hydrochloride from the Sterling-Winthrop Research Institute, U.S.A.

Phosphoglucomutase, glucose-6-phosphate dehydrogenase, and nicotine-adenosine dinucleotide phosphate used in the phosphorylase assay were purchased from Calbiochem, U.S.A. All enzymes were of grade A purity.

Statistical methods

Contractile force and heart rate effects of agonists alone and in combination with acetylcholine were determined in the same hearts, and were statistically compared using Student's *t* test for paired samples. Phosphorylase activities were compared by the *t* test for grouped data (Runyon & Haber, 1967). A *P* value of 0.05 was chosen as the level of significance.

Results

Isoprenaline in doses of 0.001 to 0.05 μg produced dose-related increases in contractile force and heart rate. Figure 1 shows the responses of guinea-pig isolated hearts measured 1 min after isoprenaline injection. Acetylcholine (0.018 μg/min) infused alone had no effect on ventricular contractility or heart rate (Figure 2). However, the positive inotropic response to all doses below 0.01 μg of isoprenaline was antagonized by infusion of acetylcholine. In contrast, the heart rate response to isoprenaline was no different in the presence or in the absence of acetylcholine. Time-response curves are presented to indicate that acetylcholine effects shown in dose-response curves are not simply a phenomenon of sampling time (Figure 2).

Salbutamol at doses of 0.25 to 1 μg produced a positive chronotropic effect without a con-

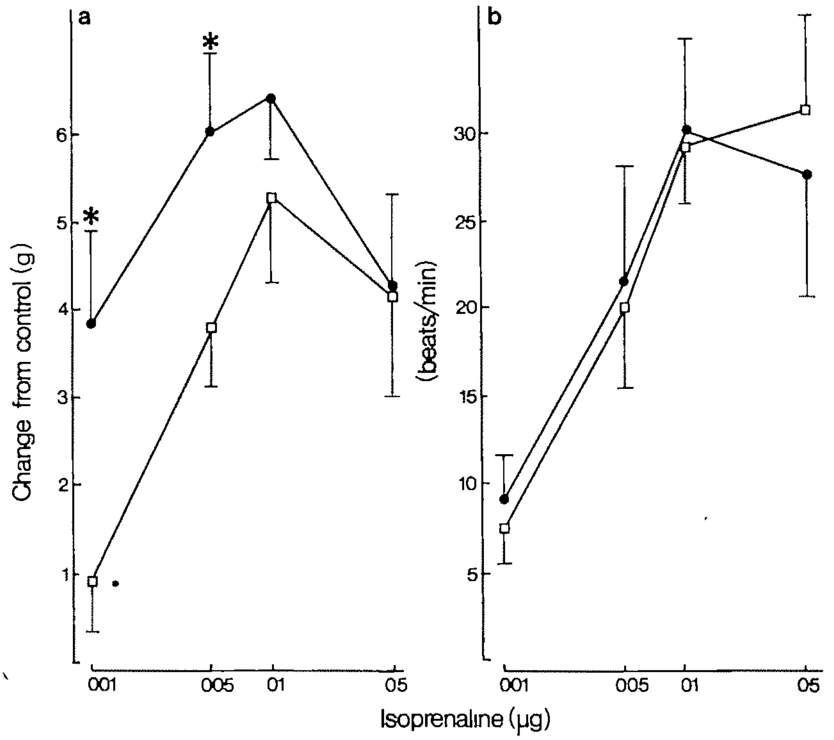

Figure 1 Contractile force (a) and heart rate (b) responses of isolated spontaneously beating heart of guinea-pig to injections of isoprenaline. Responses were measured 1 min after isoprenaline injection both before (●) and during infusion of acetylcholine, 0.018 μg/minute (□) Each point represents the mean of 4 or more experiments. In this and the following 5 figures, vertical bars represent s.e. mean and * denotes responses to agonist alone which differ significantly from the response of the same dose of agonist during acetylcholine infusion.

comitant positive inotropic effect, and 0.5 μg of salbutamol actually caused a slight, but significant decrease in contractile force. Higher doses produced increases in contractile force as well as heart rate. Acetylcholine antagonized the positive inotropic response to all except the highest dose of salbutamol used (Figures 3, 4). During acetylcholine infusion, all doses of salbutamol below 10 μg caused statistically significant decreases below control contractile force. Again, heart rate responses were unaffected by acetylcholine infusion.

Aminophylline produced dose-related increases in both contractile force and heart rate (Figure 5). Acetylcholine infusion prevented the positive inotropic response to all except the highest dose of aminophylline used. The contractile force in response to all doses of aminophylline below 3 mg was not significantly different from pre-injection control values or from the response to acetylcholine infusion alone (Figures 2, 5 & 6).

Control heart rates before administration of any dose of agonist alone and before the same dose of agonist with acetylcholine did not differ significantly. Only in the cases of the highest dose of both isoprenaline and aminophylline were control contractile force values for agonist alone significantly different from control values before agonist plus acetylcholine. However, in neither of these instances was agonist effect on contractility altered by acetylcholine.

Phosphorylase activity was determined on a separate series of guinea-pig isolated hearts frozen 60 s after drug injection. Doses of 0.005 and 0.01 μg of isoprenaline increased phosphorylase activity within 1 min after injection (Figure 7). Acetylcholine infusion antagonized the phosphorylase response to both doses of isoprenaline. The enhanced phosphorylase activity produced by salbutamol (10 μg) or by aminophylline (1 mg) was also significantly decreased by concomitant acetylcholine infusion.

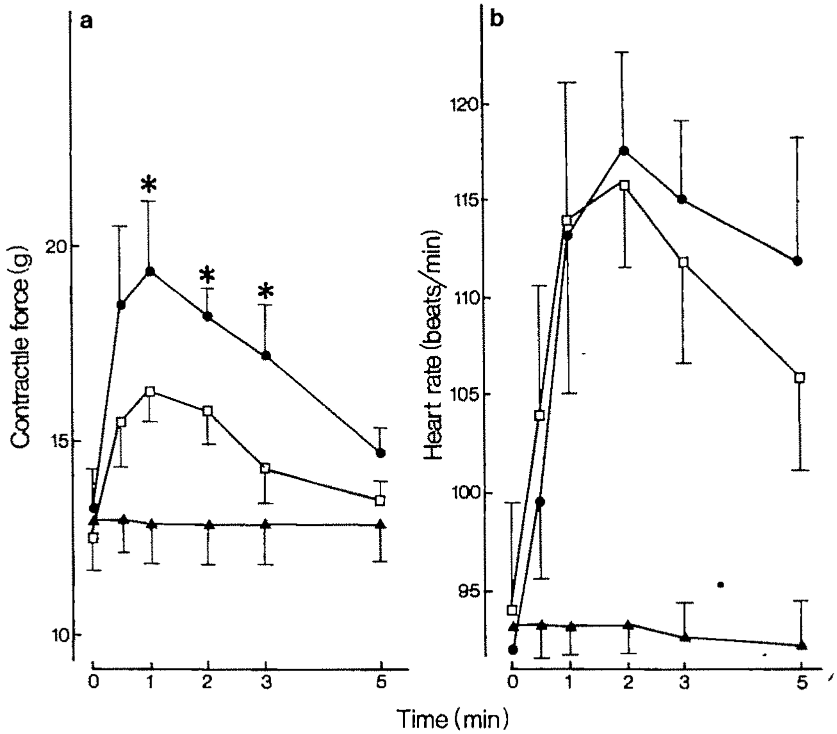

Figure 2 Time course of effects of contractile force (a) and heart rate (b) produced by isoprenaline injection (0.005 μg) in the absence (●) and the presence of acetylcholine (ACh) 0.018 μg/minute (□). (▲) shows effect of acetylcholine alone. Each point represents the mean of 4 experiments.

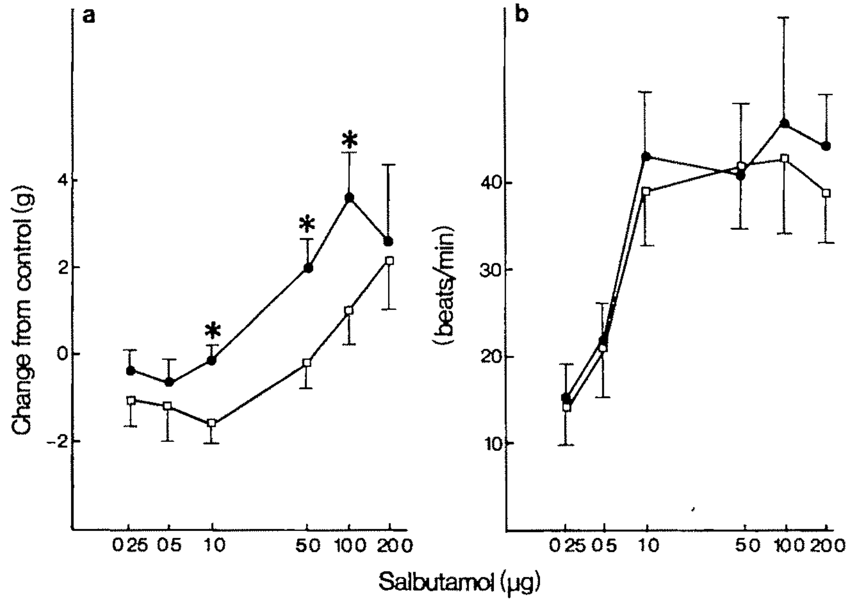

Figure 3 Effects of salbutamol alone (●) or during acetylcholine (ACh) infusion (0.018 μg/min) (□) on contractile force (a) and heart rate (b) in isolated perfused hearts of guinea-pigs. Responses were measured 1 min after salbutamol injection. Each point represents the mean of 4 or more experiments.

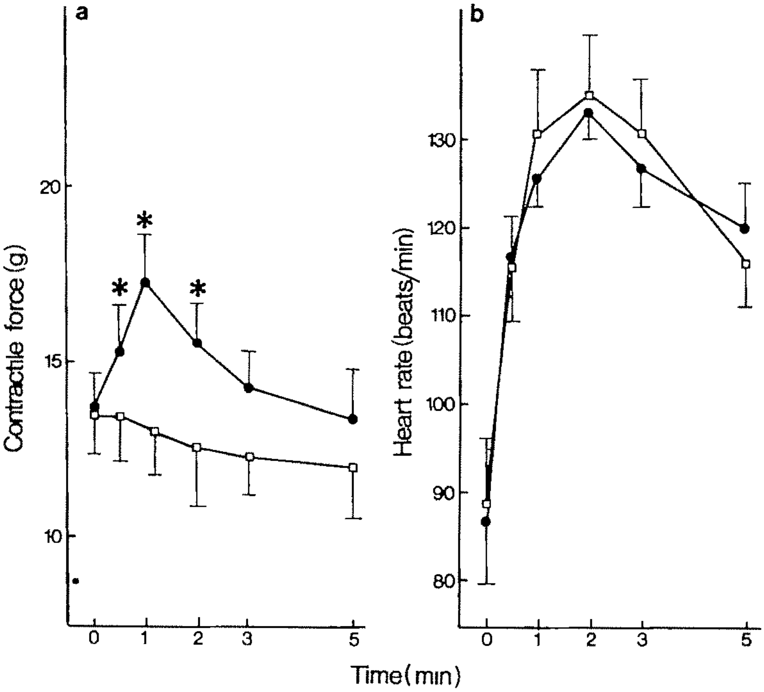

Figure 4 Time course of the effect of salbutamol (5 μg) on contractile force (a) and heart rate (b) both before (●) and during acetylcholine infusion (0.018 μg/min) (□). Each point represents the mean of 5 experiments.

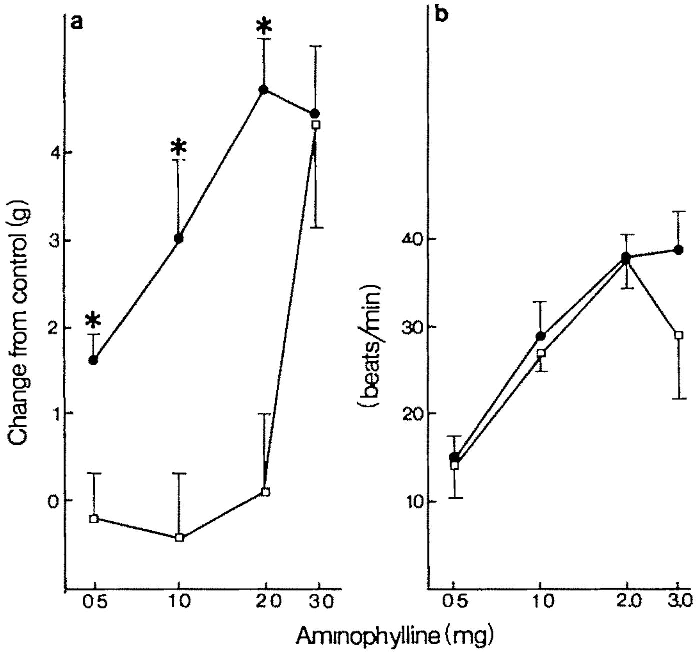

Figure 5 Effect of aminophylline injected before (●) and during acetylcholine infusion (0.018 μg/min) (□) in the guinea-pig isolated heart. Contractile force (a) and heart rate (b) responses were measured 2 min after aminophylline injection. Each point represents the mean of 4 or more experiments.

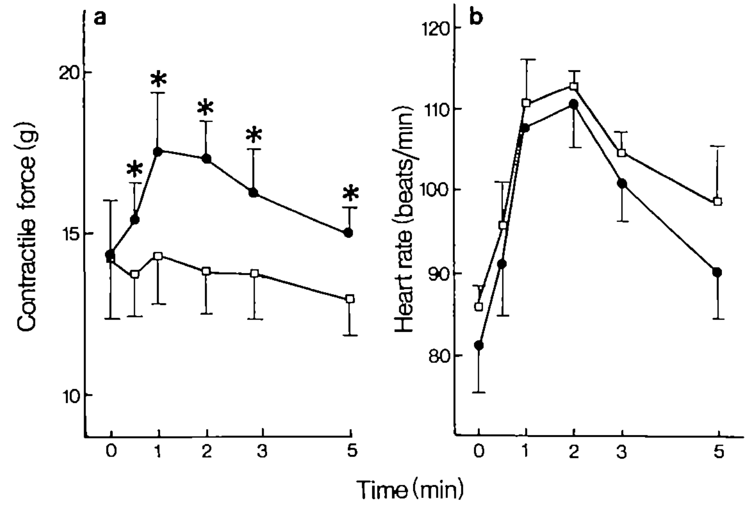

Figure 6 Time course of effect of aminophylline (1 mg) on contractile force (a) and heart rate (b) in the absence (●) and presence of acetylcholine (0.018 μg/minute) (□). Each point represents the mean of 5 experiments.

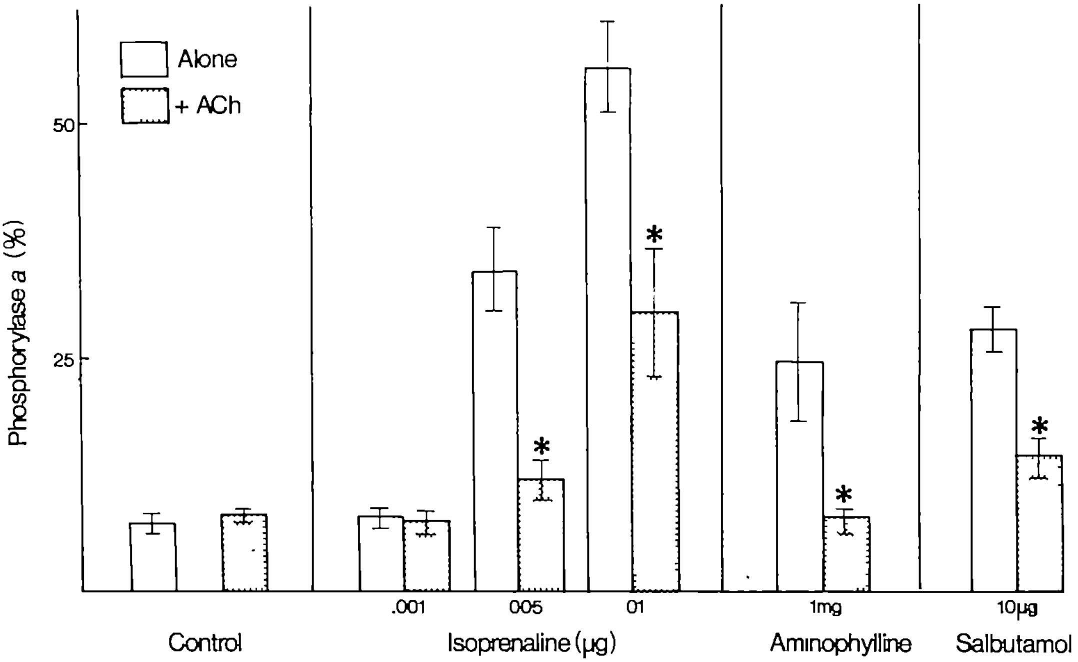

Figure 7 Effect of acetylcholine (ACh) on the activation of glycogen phosphorylase induced by isoprenaline, aminophylline and salbutamol. Results are expressed as % phosphorylase *a*

$$\left(\frac{\text{phosphorylase } a \text{ activity}}{\text{total phosphorylase activity}}\right) \times 100$$

since total phosphorylase activity was not changed from control by any treatment. Vertical bars indicate s.e. mean. * phosphorylase activity in the presence of acetylcholine which differs significantly from enzyme activity induced by the corresponding dose of isoprenaline, aminophylline or salbutamol. Each column represents the mean of 3-8 experiments.

Discussion

It has been proposed that enhancement of cardiac cyclic AMP concentration mediates the inotropic effect of β-adrenoceptor agonists and of phosphodiesterase inhibitors (Robison *et al.,* 1971). Acetylcholine has been shown to antagonize the increase in cyclic AMP produced by adrenaline or isoprenaline in various preparations (Lee *et al.,* 1971; Chamales *et al.,* 1971; Kuo *et al.,* 1971). It has further been proposed that an effect on cyclic AMP production may mediate the antagonism by acetylcholine of the cardiac effects of catecholamines (LaRaia & Sonnenblick, 1971).

The present study was performed in order to test the hypothesis that agents which antagonize the enhancement of cyclic AMP production will also antagonize the cardiac effects of catecholamines and phosphodiesterase inhibitors. Isoprenaline stimulates cardiac adenyl cyclase (Lee *et al.,* 1971) and salbutamol produces phosphorylase activation (Figure 7), presumably through cyclic AMP production. Both these agents stimulate cardiac rate and force, but salbutamol causes a positive inotropic response only with doses that produce a maximal increase in heart rate, whereas isoprenaline causes a positive inotropic response in doses which produce less than 15% increase in heart rate. From the present experiments a ratio between the dose causing a standard change in heart rate and the dose causing a standard change in contractile force was calculated for each agonist. These ratios are shown in Table 1. The large differences among the ratios for the three agonists suggest that the rate and contractility responses are not mediated by a single intermediate.

Table 1 Ratio between dose required to produce 25% increase in heart rate (HR ED_{25}) and dose which produces 25% increase in contractile force (CF ED_{25})

Agonist	$\frac{HR\ ED_{25}}{CF\ ED_{25}}$
Isoprenaline	4.808
Salbutamol	0 039
Aminophylline	0.636

The concentration of acetylcholine used in these experiments had no measurable effect on either rate or contractile force, but did antagonize the effect of all three agonists on contractility. It has previously been shown that only concentrations of acetylcholine which produce a bradycardia can antagonize the chronotropic effect of adrenaline (Chamales *et al.,* 1971).

The negative inotropic effect seen when salbutamol was administered with acetylcholine, or when 0.5 μg of salbutamol was administered alone appears to have been a function of the different inotropic and chronotropic potencies of this agonist. These decreases in contractile force occurred primarily as increases in end-diastolic resting tension, rather than decreases in systolic tension, and could have resulted from increases in rate without changes in time to peak tension. This response to 0.5 μg of salbutamol could reflect the interaction of a sub-threshold inotropic dose with an effective chronotropic dose, and with acetylcholine could result from antagonism of inotropic, but not chronotropic effects.

In the present experiments the positive inotropic responses and phosphorylase activation induced by isoprenaline, aminophylline and salbutamol were antagonized in parallel by acetylcholine. A role for cyclic AMP in the production of these responses cannot be ruled out on the basis of the present data. However, the differences among the potency ratios for the three agonists, and the lack of effect of acetylcholine on the chronotropic response suggest that heart rate changes are not mediated solely by cyclic AMP. Although cyclic AMP participation in the heart rate response cannot be eliminated on the basis of these experiments, the data indicate that other factors must be involved.

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A CHEMOTACTIC ROLE FOR PROSTAGLANDINS RELEASED FROM POLYMORPHONUCLEAR LEUCOCYTES DURING PHAGOCYTOSIS

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1 Prostaglandin E_1 is chemotactic at concentrations down to 10 ng/ml for rabbit polymorphonuclear (PMN) leucocytes. Prostaglandins E_2 and $F_{2\alpha}$ have little or no chemotactic effect at concentrations up to 10 μg/ml.

2 Washed PMN leucocytes produce a chemotactic agent during phagocytosis, but not in the presence of indomethacin (28 μM).

3 Phagocytosing PMN leucocytes produce up to ten times as much prostaglandin as do resting cells. Some of this is prostaglandin E_1 as judged by thin layer chromatography and differential bioassay. This prostaglandin production by PMN leucocytes is abolished by indomethacin (28 μM).

4 Ultrasonicated suspensions of PMN leucocytes produce prostaglandin from arachidonic acid. This synthesis is inhibited by indomethacin.

5 Homogenates of PMN leucocytes which have been pre-incubated with bacteria for 30 min show more prostaglandin synthetase activity than homogenates from PMN leucocytes which have not been exposed to bacteria.

6 It is concluded that in some forms of inflammation, prostaglandin E_1 may play a controlling role in cellular migration.

7 PMN leucocytes may contribute to the generation of prostaglandins found in some inflammatory lesions.

Introduction

Some inflammatory exudates contain prostaglandins in concentrations high enough to mediate certain features, such as oedema and erythema, of the inflammatory response (Willis, 1969; Crunkhorn & Willis, 1971). Several non-steroid anti-inflammatory drugs at therapeutic concentrations inhibit prostaglandin synthesis in various systems (Vane, 1971; Smith & Willis, 1971; Ferreira, Moncada & Vane, 1971). Thus, prostaglandins may make a significant contribution to certain forms of inflammation. Although many different tissues release prostaglandins when suitably stimulated, there is evidence that in at least one form of experimental inflammation, immunogenic uveitis in rabbits (Eakins, Whitelocke, Perkins, Bennett & Unger, 1972) the source of the prostaglandin is the polymorphonuclear (PMN) leucocytes, cells in which we have demonstrated the capacity to produce prostaglandins during phagocytosis (Higgs & Youlten, 1972).

Prostaglandin E_1 is chemotactic for rabbit PMN leucocytes (Kaley & Weiner, 1971). The present investigation explores the possibility that prostaglandins released from PMN leucocytes might have a controlling role in attracting more cells to sites where phagocytosis is occurring. We have also investigated the amounts and types of prostaglandin released from PMN leucocytes *in vitro* to see if they are likely to make an important contribution *in vivo*. Some of these results have been communicated to the British Pharmacological Society (Higgs & Youlten, 1972) and to the Physiological Society (McCall & Youlten, 1973).

Methods

Harvesting of polymorphonuclear leucocytes

PMN leucocytes were obtained by the method of Hirsch & Church (1960). Six adult New Zealand White rabbits were each used in rotation once every 7-10 days. A sterile peritonitis was induced by the intra-peritoneal administration of 200 ml 0.9% w/v sterile NaCl solution (saline) containing 0.1% w/v glycogen. Four hours later the peritoneal cavity was washed out through a plastic cannula with 100 ml Hanks basic tissue culture medium, and the contents collected into siliconized glass tubes. A total cell count was made on one sample (0.1 ml) and another (0.1 ml) stained for a differential cell count. The remainder was centrifuged at 600 rev/min (52 x *g*) for 10 min, the supernatant was poured off and the cells resuspended to the required concentration in Hanks solution enriched with glucose (0.56 mM) and bovine serum albumin (100 μg/ml). Total cell counts varied between 1 x 10^6/ml and 15 x 10^6/ml; by differential count, over 98% of the cells were PMN leucocytes. On the few occasions when there was erythrocyte contamination or the viability (measured by the exclusion of trypan blue dye, 10 mg/ml) was below 95%, the cells were discarded.

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Chemotaxis

Chemotactic activity was measured with a perspex chamber separated by a Millipore filter (SCWPO 1300 pore size 8 μm) into two compartments (Boyden, 1962). Test solutions or suspensions were placed in the lower compartment, and cell suspensions in the upper one. After incubation at 37° C for 3 h, the filter was stained and its lower surface examined for stained cells, the number of cells being counted in each of nine randomly selected high power fields. This number is taken as an index of chemotactic attraction by the contents of the lower compartment for the cells in the upper compartment. In control experiments, the suspected chemotactic substance was added in equal concentrations to both compartments.

Prostaglandin production by phagocytosing cells

PMN leucocytes were resuspended in enriched Hanks medium to a concentration of 3.5-8.0 x 10^6 cells/ml. A portion (20 ml) of this suspension was put in each of two wide-necked tubes and to one killed bacteria (100/cell) were added. The tubes were lightly stoppered and incubated in a water bath at 37° C for up to 3 hours. The cells were then centrifuged (52 x *g*; 10 min) and the supernatants collected. Each sample was acidified to pH 3 with 0.1 N HCl and extracted twice with equal volumes of ethyl acetate. The two extracts were pooled and evaporated to dryness under reduced pressure. For bioassay of prostaglandins, the residue was dissolved in 1 ml Krebs bicarbonate solution and a portion of this injected over a rat stomach fundus strip (Vane, 1957), superfused at 5 ml/min with Krebs bicarbonate solution at 37° C containing a mixture of antagonists to prevent the action of acetylcholine, 5-hydroxytryptamine, histamine or the catecholamines (Gilmore, Vane & Wyllie, 1968). For thin-layer chromatography, the residue was dissolved in 5 ml ethanol/water (2:1, v/v) and partitioned four times against petroleum ether (b.p. 40-60° C). The aqueous ethanol phase was evaporated under reduced pressure, the residue redissolved in 0.25 ml methanol and applied to thin layer silica plates along with prostaglandin standards. The plates were developed in the AI or AII system (Gréen & Samuelsson, 1964) and divided into strips of 1 cm, which were scraped into tubes and eluted with 1 ml Krebs solution. After centrifugation, the resulting supernatants were bioassayed against prostaglandin standards on rat stomach fundus strip, chick rectum and rat colon. Small quantities (0.36 ng) of [5,6-(n)^{3}H]-prostaglandin E_1 (Radiochemical Centre, Amersham) were added to the samples before extraction and 0.1 ml aliquots of the final eluants were counted in a Packard Tricarb Liquid Scintillation system.

Prostaglandin synthesis by cell sonicates

PMN leucocytes were resuspended in 100 mM cold phosphate buffer (pH 7.4) at a concentration of 50-100 x 10^6 cells/ml. They were ultrasonicated at 20,000 Hz for 2 min and the resulting homogenate was used as the enzyme preparation. Prostaglandin synthetase activity was measured by the method of Flower, Gryglewski, Herbaczynska-Cedro & Vane (1972). The reaction mixture contained arachidonic acid (20 μg), reduced glutathione (100 μg), hydroquinone (10 μg) and cell homogenate (0.5 ml), and was made up to 2.0 ml with phosphate buffer. The reaction was started by adding the enzyme to the other components and samples were taken immediately after this addition (zero-time) and after 30 min incubation at 37° C, and the reaction was stopped by boiling for 45 seconds.

The amount of prostaglandin-like material produced by the enzyme system was assayed in terms of prostaglandin E_2 on a rat stomach strip superfused as previously described, with the addition of indomethacin (1 μg/ml) to prevent intrinsic prostaglandin formation by the tissue (Eckenfels & Vane, 1972).

In other experiments, PMN leucocytes were resuspended to a concentration of 5 x10^6 cells/ml in modified Hanks solution and incubated at 37° C for 30 min, either alone or in the presence of *Bordetella pertussis* in a ratio of 1000 bacteria per cell. The suspensions were then centrifuged

($52 \times g$) for 10 min, the supernatants poured off and the cells resuspended in a small volume of cold phosphate buffer and ultrasonicated. The resulting homogenates were then tested as above for their prostaglandin synthesizing activity. In each sample, the total protein was measured by the biuret method.

Materials

Substances used were Hanks TC medium (Difco), shellfish (type II) or rabbit liver (type III) glycogen (Sigma), chemically killed *Bordetella pertussis* (Per/Vac, Burroughs Wellcome), indomethacin (Merck Sharp & Dohme), prostaglandins E_1, E_2 and $F_{2\alpha}$ (Upjohn) and [5,6(n)^{3}H]-prostaglandin E_1 (Radiochemical Centre, Amersham).

Results

Chemotactic activity of prostaglandins

When the lower compartment of the Boyden chamber contained sterile saline, after 3 h incubation, 20-75 cells were counted in 9 high power fields on the lower surface of the Millipore filter. The cell viability, tested by the exclusion of trypan blue dye at the end of each incubation was always greater than 90%.

Prostaglandin E_1 (1 µg/ml) was always chemotactic compared with the saline controls (Table 1). The mean increase was 3.6-fold with a range of 2.2-5.8. The differences between the means were significant ($P < 0.02$ by Student's paired t test).

Table 2 shows the chemotactic response induced by different concentrations of prostaglandins E_1, E_2 and $F_{2\alpha}$ from 10 ng/ml to 10 µg/ml. Prostaglandin E_1 appeared to have chemotactic activity at all concentrations tested. The effects of prostaglandins E_2 and $F_{2\alpha}$ were comparatively small. At 1 µg/ml, for example, prostaglandins E_2 and $F_{2\alpha}$ caused mean increases of only 1.1 and 1.2 times the control values. In five experiments, no increase in cell migration above control was seen when the upper compartment contained the same concentration of prostaglandin E_1 as the lower.

Chemotactic effect of phagocytosing cells

The chemotactic effect of phagocytosing cells was measured in 7 experiments. Dead bacteria were added to a cell suspension in the lower compartment in a ratio of 100 bacteria per cell. The chemotactic activities of the cell suspension alone and of the bacteria alone were also measured (Table 3). The presence of a cell suspension in the lower compartment did not produce a result significantly different ($P > 0.1$) from the saline control. The dead bacteria alone produced significantly less cell migration than was found with saline ($P < 0.05$). The cells and bacteria together produced a significant ($P < 0.02$) chemotactic response, (mean 3.4 times control (cells only) values, range 1.9-4.5).

In the presence of indomethacin (10 µg/ml - 28 µM) in three experiments cells and bacteria together had only slightly more chemotactic effect than saline controls. At a higher

Table 1 Chemotactic effect of prostaglandin E_1 (PGE_1) on polymorphonuclear (PMN) leucocytes

Expt no.	*Lower chamber contents*		*Ratio of PGE_1/saline*
	Saline	*PGE_1*	
1	75	434	5.8
2	51	112	2.2
3	30	95	3.2
4	35	164	4.7
5	36	111	3.1
6	35	97	2.8
7	20	102	5.1
8	31	121	3.9
9	24	58	2.4
10	46	131	2.8
	$P < 0.02$ (paired t test)		mean = 3.6

Results of 10 experiments using Boyden chambers with different PMN leucocyte suspensions (3×10^6/ml) in the upper compartment and prostaglandin E_1 (1 µg/ml) or saline in the lower compartment. Figures give total cell counts in 9 high power fields on the lower surface of the filter.

concentration of indomethacin (280 µM) there was slightly less cell migration than in controls (2 experiments). Indomethacin did not affect the cell migration in the bacteria-free saline controls.

Phagocytosis of the bacteria by the PMN leucocytes was demonstrated by the uptake of ^{125}I-labelled human serum albumin (HSA) (Chang, 1969) from the incubation medium. PMN leucocytes incubated without bacteria showed no increase in uptake of [^{125}I]-HSA over a 50 min period, while the [^{125}I]-HSA content of the cells incubated with bacteria (100/cell) rose over the same period to over three times the initial value

Prostaglandin production by polymorphonuclear leucocytes during phagocytosis

Several substrates were tried for phagocytosis: glass beads were too large for the PMN leucocytes to ingest. Microscopy showed that the cells tended to spread over the surface of the beads. Latex beads aggregated in solution at 37° C, and the aggregated particles were too large for successful phagocytosis. In all experiments described here *Bordetella pertussis* was used. Microscopy confirmed that these were ingested by the PMN leucocytes.

Table 2 Chemotactic effect of different prostaglandins (PG) on polymorphonuclear (PMN) leucocytes

	10 ng/ml	*100 ng/ml*	*1 µg/ml*	*10 µg/ml*
PGE_1	1.62 ± 0.25	2.76 ± 0.42	3.09 ± 0.28	2.81 ± 0.63
n	7	7	22	10
P	<0.05	<0.01	<0.001	<0.02
PGE_2		0.45 ± 0.14	1.1 ± 0.55	1.2
n		7	5	2
P		<0.02	NS	
$PGF_{2\alpha}$		0.63 ± 0.2	1.24 ± 0.16	1.2
n		5	7	3
P		NS	NS	

Results using Boyden chambers with PMN leucocytes (3 x 10^6/ml) in the upper compartment and various concentrations of different prostaglandins in the lower compartment. Results are expressed as the ratio, experiment/control ± s.e.mean.

P values are those from Student's paired *t* test: NS indicates $P > 0.1$.

Table 3 Chemotactic effect of polymorphonuclear (PMN) leucocyte–bacteria mixtures

Expt. no.	*Contents of lower compartment*			
	Saline	*Cell suspension*	*Dead bacteria*	*Cell suspension + dead bacteria*
1	19	22	18	35
2	54	37	58	155
3	17	22	8	76
4	12	5	3	24
5	19	21	7	51
6	12	26	5	38
7	75	283	16	317

NS

$P < 0.05$

$P < 0.02$

Results (total cells in 9 high power fields) of 7 experiments using Boyden chambers with PMN leucocyte suspensions (3 x 10^6/ml) in the upper compartment, contents of the lower compartment as stated.

P values are those from Walsh test as shown for comparison with appropriate controls. NS . $P > 0.1$.

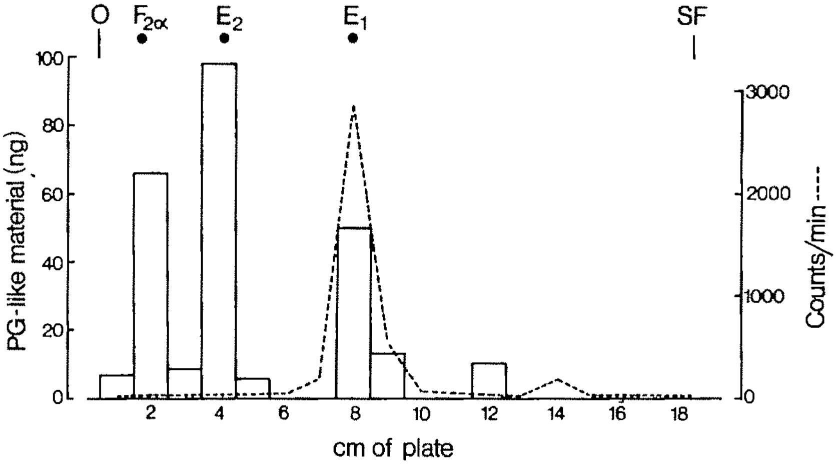

Figure 1 AII thin-layer chromatography of prostaglandin (PG)-like material extracted from the supernatant of cell suspensions incubated with killed bacteria. The spots show the positions of standards of authentic prostaglandins $F_{2\alpha}$, E_2 and E_1. The broken line shows the radioactivity in successive samples eluted from the plate, to which ^{3}H-labelled prostaglandin E_1 had been applied with the sample. The histogram shows biological activity expressed as prostaglandin E_2 equivalents assayed on the rat stomach strip.

Initially the bacteria were used at a concentration of 1000 bacteria per cell. In these conditions there was negligible (less than 0.3 ng/10^7 cells) production of a prostaglandin-like material at zero-time, but production rose to 7.1 ng/10^7 cells (range, 4.3-11.0) after 1 h of incubation. In 5 experiments, the amount of prostaglandin-like material after 2 h ranged from 2.4 to 17.3 ng/10^7 cells (mean, 8.4). In general, amounts of prostaglandin-like material rose with time to a peak of over 30 ng/10^7 cells at 3 h, after which the level fell, presumably because destruction had overtaken release.

The amount of prostaglandin-like material in the supernatant also varied with the bacteria-cell ratio. When this ratio was between 0.1 and 10 there was a negligible amount of prostaglandin-like material found at 1 or 2 h (1 ng/10^7 cells). Above this ratio the level rose with the bacteria-cell ratio reaching a maximum at 1000, (range 4.3-17 ng/10^7 cells). There was no further increase at higher ratios.

In the presence of indomethacin (28 μM) the level of prostaglandin-like material in cell-bacteria mixtures was not significantly greater than in bacteria-free cell suspensions (5 experiments).

Extracts of supernatants of cell suspensions incubated with bacteria were subjected to thin-layer chromatography, using the AI system and measuring the activity eluted from the zones of the plate against prostaglandin E_2 standards on superfused rat stomach fundus strip. Sixty per cent of the recoverable activity chromatographed with prostaglandin E_2, 25% with $F_{2\alpha}$, and the remainder was located in a position between prostaglandin $F_{2\alpha}$ and the origin. Further characterization of extracted prostaglandins using the AII system showed that 39-80% of the prostaglandin E-like activity corresponded to authentic prostaglandin E_1, both in its R_F value and in its biological activity on the three assay tissues. The rest of the activity had the chromatographic characteristics of prostaglandins E_2 and $F_{2\alpha}$.

Extracts from several experiments were combined to attain enough activity for accurate bioassay. [^{3}H]-prostaglandin E_1 was added before extraction to check that the mobility of the prostaglandin was not altered by the extraction procedure. Results of one of the chromatograms developed in the AII system are shown in Figure 1.

Prostaglandin synthesis by cell sonicates

In these experiments, the synthetic activity of the sonicates is expressed as ng of prostaglandin E_2-like material produced per mg protein in the sonicates. There was always some prostaglandin-like material present in the samples at zero-time; this is probably due to production during sonication. The activity found at zero-time was subtracted from that in subsequent samples to give a value representing net synthesis.

Without addition of substrate (2 experiments), there was a net synthesis after 30 min of 0.1 ng/mg protein. This synthesis rose in the presence of di-homo-γ-linolenic acid (10 μg/ml) to

Table 4 Effect of indomethacin on the synthesis of prostaglandin-like material by cell sonicates

Final conc. (µM) of indomethacin in incubation mixture	*Prostaglandin-like material (ng/mg protein)*	n
Control (no indomethacin)	3.0 ± 1.2	5
2.8	1.2	2
28	0.5 ± 0.4	4
280	0.2	3

Results are shown as the mean with s.e.mean.

0.5 ng/mg (2 experiments) and in the presence of arachidonic acid (10 µg/ml) (5 experiments) to 3.6 ± 1.2 ng/mg protein (mean ± s.e.mean).

In 8 experiments, cells were incubated with or without bacteria for 30 min before sonication. The cell sonicate from cells incubated without bacteria produced, after addition of arachidonic acid, 5.6 ± 1.8 ng prostaglandin E_2-like material/mg protein. Cells which had been preincubated with bacteria (100/cell) before sonication produced 10.6 ± 2.7 ng/mg protein. A Student's paired *t* test showed this increase to be significant ($P < 0.05$). Bacteria alone incubated for 30 min before sonication did not produce any prostaglandin-like material, and addition of bacteria to a cell sonicate immediately before incubation with substrate did not increase the prostaglandin production.

Indomethacin (0.2 ml of 1, 10 or 100 µg/ml in 20% absolute ethanol, 80% buffer) was added to the incubation mixture to give a final concentration of 2.8, 28 or 280 µM and the synthesis of prostaglandin-like material by cell sonicates, obtained from cells which had not previously been incubated, was measured. Increasing the concentration of indomethacin caused a progressive decrease in prostaglandin synthesis (Table 4). An equal amount of absolute ethanol added to the sonicates before incubation had no effect on the production of prostaglandin-like material. Addition of indomethacin to give a final concentration of 28 µM before the sonication of the cell suspension also caused a decrease in prostaglandin synthesis, to 0.1 ng/mg (mean of 3 experiments).

Discussion

Homogenates of rabbit PMN leucocytes have the capacity to synthesize prostaglandin from endogenous or exogenous precursors. The synthetase system of these cells is, like that of other tissues, inhibited by indomethacin. The finding that homogenates from cells which have been preincubated with bacteria have greater synthetic ability than homogenates from bacteria-free controls, suggests that the prostaglandin synthesis occurring during phagocytosis is not due only to increased substrate availability, or to cell membrane damage, but may involve a change in cellular enzyme activity during phagocytosis.

Rabbit peritoneal PMN leucocytes produce and release prostaglandins, including prostaglandin E_1, when ingesting dead bacteria. Furthermore, the amounts produced *in vitro* seem adequate to account for the levels of prostaglandins found in inflammatory exudates. Ten million cells produced up to 30 ng of prostaglandin-like activity in 3 hours. The concentrations attained are also adequate to account for the chemotactic influence of phagocytosing cells *in vitro* discussed below. Our findings strongly support the view of Movat, MacMorine & Takeuchi (1971) that the vascular permeability-increasing agent released from rabbit PMN leucocytes during phagocytosis is a prostaglandin. Experimental immunogenic uveitis has been studied in rabbits by Eakins *et al.* (1972). Prostaglandin E_1 was found in inflamed but not in normal rabbit eyes. Normal eye tissues contained only prostaglandins E_2 and $F_{2\alpha}$. The appearance of prostaglandin E_1 coincided with the invasion of the ocular tissues and aqueous humour by PMN leucocytes. These findings are consistent with our detection of E_1 among the prostaglandins produced by phagocytosing PMN leucocytes, and support our view that in some forms of inflammation the source of the prostaglandins found may be the PMN leucocytes.

We have confirmed the chemotactic activity of prostaglandin E_1 for washed (plasma-free) PMN rabbit leucocytes, first described by Kaley & Weiner (1971). We have further shown that this effect is concentration-dependent and detectable down to 10 ng prostaglandin E_1/ml, well below the total prostaglandin concentrations which have been reported in inflammatory exudates (80 ng/ml, Willis, 1969; 140 ng/ml, Eakins *et al.*, 1972). It is thus possible that the prostaglandins in inflammatory lesions could have a chemotactic effect *in vivo* on PMN leucocytes. The effect of prostaglandins on other motile cells such as macrophages was not studied, but seems worth investigating.

Phagocytosing PMN leucocytes, but not cells or bacteria alone, have a chemotactic effect on other cells. This chemotactic activity of cell-bacteria mixtures is greatly reduced or abolished in the presence of indomethacin (28 μM or 280 μM respectively). This supports the view that the effect may be mediated by a prostaglandin since at these concentrations indomethacin inhibits prostaglandin synthesis in such mixtures. Reports that indomethacin decreased cell motility (Phelps, 1969) are based on an experimental system in which urate crystals were used as a chemotactic stimulus. In the light of our findings the results can be re-interpreted as abolition by indomethacin of a prostaglandin-mediated chemotactic effect associated with phagocytosis of the crystals by the first cells reaching them.

It should be noted that the present studies were performed with washed cells, and that, *in vivo*, other chemotactic agents such as activated complement factors, may be more important than the prostaglandins. In addition, phagocytosing cells may release other substances which could act as chemotactic agents. This may explain why non-steroid anti-inflammatory drugs are unable to abolish fully the cellular invasion in inflammatory lesions.

Influences other than phagocytosis may also induce prostaglandin release from PMN leucocytes. Thrombin, for instance, does so (our unpublished observation) and this or other mechanisms may be concerned in chemotaxis in the absence of phagocytosis.

Moncada, Ferreira & Vane (1974) have shown that 3 h after carrageenin injection into the dog knee joint, prostaglandin E_2 can be detected in the synovial fluid. In human arthritis, however, where the PMN leucocyte concentration in synovial fluid is high, the predominant prostaglandin is E_1 (Higgs, Vane, Hart & Wojtulewski, 1974). Since PMN leucocyte migration begins in carrageenin-induced oedema 2-3 h after injection (Di Rosa, Papadimitriou & Willoughby, 1971), it seems possible that the source of the prostaglandin E_1 is the PMN leucocytes. In a similar model (Phelps & McCarty, 1967) indomethacin treatment resulted in decreased fluid exudate and reduced PMN leucocyte infiltration.

It is concluded that in some forms of inflammation, PMN leucocytes may be the source of the prostaglandin found. Such prostaglandin production, particularly that of prostaglandin E_1 could have a specific role in inducing or enhancing local migration of phagocytic cells, as well as in the modulation of the inflammatory effects of other mediators. Such a system would have the obvious advantage of encouraging invasion by phagocytic cells only as long as there was material capable of being phagocytosed present. It could not, alone, explain how such cellular migration is initiated.

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ENHANCED INACTIVATION OF PROSTAGLANDIN E_2 BY THE RABBIT LUNG DURING PREGNANCY OR PROGESTERONE TREATMENT

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1 The inactivation of prostaglandin E_2 by the rabbit lung was estimated *in vivo* by comparing its depressor potency following intravenous and intra-aortic injections, and *in vitro* by measuring the rate of disappearance of smooth muscle stimulating activity when the prostaglandin was incubated with high speed supernatant fractions from lung homogenates.

2 The ability of the lung to inactivate prostaglandin E_2 *in vivo* increased gradually throughout pregnancy, and then decreased rapidly during the three days post-partum.

3 An increased lung inactivation was also seen in pseudopregnant (day 12) rabbits, and in non-pregnant rabbits treated with progesterone for 12 days. A further increase occurred when progesterone treatment was prolonged to 26 days.

4 Treatment with oestradiol monobenzoate or cortisol for 12 days, and deprivation of ovarian hormones for 14-17 days by ovariectomy, were without effect on the lung inactivation of prostaglandin E_2.

5 The *in vitro* experiments revealed a striking increase in the activity of lung prostaglandin metabolizing enzymes during pregnancy.

6 The results are discussed in relation to the hormonal changes occurring during pregnancy, and it is suggested that an enhanced lung inactivation of prostaglandins might have an important protective function at this time.

Introduction

The lungs of several animal species are extremely efficient at removing prostaglandins of the E and F series from the circulation (Ferreira & Vane, 1967; Horton & Jones, 1969). Thus, they reduce the passage of prostaglandins from the venous blood into the arterial circulation, where they would otherwise have marked effects on the cardiovascular system. This protective function might be expected to be particularly important at the time of parturition, when there is evidence that uterine activity is aided by endogenous prostaglandins (Waltman, Tricomi & Palav, 1973; Williams, Sneddon & Harney, 1974). However, in humans, it is clear that the lungs do not inactivate all the prostaglandin presented to them during parturition, because the concentrations of prostaglandins E_2 and $F_{2\alpha}$ in ante-cubital venous blood increase during labour, and the levels fluctuate in parallel with the uterine contractions (Karim, 1968; Hertelendy, Woods & Jaffe, 1973). Thus, it seems that either the inactivating mechanism is swamped by large amounts of prostaglandins in the venous blood, or that changes occur during pregnancy which facilitate the passage of prostaglandins through the lungs unchanged. To obtain information about the metabolism of prostaglandins during pregnancy we have studied the rate of inactivation of prostaglandin E_2 by the lungs of non-pregnant, pregnant and post-parturient rabbits.

A preliminary account of some of these results was given at a meeting of the British Pharmacological Society (Bedwani & Marley, 1974).

Methods

Animals

Mature female Dutch rabbits were used throughout these experiments. Where indicated, pseudopregnancy was induced by an intravenous injection of human chorionic gonadotrophin (HCG, 75 iu). The day of the injection of HCG or of mating was called day 1.

In vivo *experiments*

The method used to measure the inactivation of prostaglandin E_2 by the rabbit lung *in vivo* was in principle that described by Horton & Jones (1969). The loss of vasodepressor activity following a single passage through the lungs was measured by comparison of the depressor potencies of intravenous and intra-arterial injections of the prostaglandin.

Rabbits were anaesthetized with intravenous pentobarbitone sodium. Blood pressure was recorded from a femoral artery. Intra-arterial injections of prostaglandin E_2 were given through a catheter which had been inserted into the right carotid artery and advanced into the ascending aorta. Intravenous injections were given high into the vena cava through a catheter advanced via the femoral vein. Prostaglandin E_2 was dissolved in 0.9% w/v solution NaCl (saline) and each injection was washed in with 0.4-0.8 ml of the solvent.

Responses to a range of intra-arterial and intravenous doses of standard stock solutions of the prostaglandin were obtained. Two doses which gave clearly defined but submaximal depressor responses, usually $<$ and $>$20 mmHg (1 mmHg = 133 Pa) were selected for each route, the ratio between the doses being the same. Then, a fresh dilution suitable for the intra-arterial injections was prepared from the solution to be used for the intravenous injections. The four doses selected were each given 3 to 5 times, following a Latin square design and a dose cycle of 10 minutes. The dose required to cause a 20 mmHg fall in diastolic blood pressure was measured graphically for each route, and the dose-ratio (i.v. dose/i.a. dose) was calculated. This was taken as a measure of the degree of inactivation of the prostaglandin by the lung.

In vitro *experiments*

The metabolism of prostaglandin E_2 by lung enzymes *in vitro* was investigated by measuring its rate of inactivation on incubation with crude enzyme preparations from non-pregnant and pregnant (day 26) rabbits. Inactivation was measured as loss of smooth muscle stimulating activity, since the lung metabolites of prostaglandin E_2 have little activity on smooth muscle (Piper, Vane & Wyllie, 1970).

Lungs, taken from freshly killed animals, were washed thoroughly and homogenized in ice-cold Bucher medium (K_2HPO_4, 0.072 M; KH_2PO_4, 0.02 M; $MgCl_2$, 3.6 mM; nicotinamide, 0.0276 M) to give a tissue : medium ratio of 1 : 9. The homogenate was centrifuged at 1400 *g* for 3 min, after which the supernatant was centrifuged at 100,000 *g* for 1 hour. A 1 ml aliquot of the final supernatant was diluted to 22 ml with Bucher medium and NAD^+ was added to give a final concentration of 2 mM. This crude enzyme preparation was thus equivalent to a dilution of the tissue of 1 part in 220.

To measure enzyme activity, a 10 ml aliquot of this enzyme preparation was incubated at 30° C and the reaction was started by adding 10 μg of prostaglandin E_2. Aliquots (1 ml) of the reaction mixture were removed at the appropriate times and added to tubes containing 0.1 ml of 1 N HCl, a procedure found to inactivate the enzyme preparation. The prostaglandin E_2 in each aliquot was then extracted with 2 x 3 vol. ethyl acetate, and the extracts were evaporated to dryness at 42° C. The residue was dissolved in water, and assayed on the rat stomach strip (Vane, 1957) against prostaglandin E_2 standards.

The efficiency of this extraction procedure was estimated in each experiment by measuring the amount of prostaglandin E_2 which could be recovered from 1 ml of the enzyme preparation kept at 0° C, immediately after the addition of 1 μg of the prostaglandin. The recovery was 93.7% ± 6.0 (mean ± s.e., $n = 6$) and the results have been corrected accordingly.

Aliquots of the enzyme preparation were also taken for determination of total protein, by the method of Lowry, Rosebrough, Farr & Randall (1951), to see if there was any detectable difference between preparations from the non-pregnant and pregnant rabbits.

Results

In vivo *experiments*

Intra-arterial and intravenous injections of prostaglandin E_2 produced rapid falls in diastolic blood pressure which were maximal after 10-16 s and 16-32 s respectively. Recovery was complete within 4 min in most cases, but after an unusually large fall in blood pressure the recovery was sometimes not complete until 7 minutes. In no case was a fall in blood pressure followed by a pressor response, and therefore there was no evidence that the doses used caused catecholamine release.

The log dose-response curves for the prostaglandin did not show any obvious deviations from linearity, but in the majority of experiments the curve for the intravenous injections was significantly steeper than that for the intra-arterial injections ($P < 0.05$, Student's t test). Thus, the possibility existed that a part of the fall in blood pressure caused by the intravenous doses was due to an action on sites not immediately available to the intra-arterial doses, for example in the heart or lungs. However, large falls in blood pressure caused by prostaglandin E_2 given intravenously were not accompanied by changes in the heart rate (determined from the blood pressure record at a chart speed of 2.5 mm s^{-1}), and were unaffected by bilateral vagotomy (3 experiments). Thus, the intravenous doses had no direct chronotropic effect, nor did they elicit pulmonary or cardiac depressor reflexes.

Another possibility was that, following the intra-arterial injections, a bolus of prostaglandin E_2 might have passed up the left carotid artery to reach the central nervous system and exert a pressor effect (cf. Carlson & Orõ, 1966). However, the slope of the intra-arterial dose-response curve was unaffected by clamping the left carotid artery (2 experiments), showing that this was not the case.

Our finding that the intra-arterial and intravenous dose-response curves were not parallel meant that the potency ratio between the two routes could not be calculated by the standard methods applicable to 'four-point' assays. For this reason, comparisons of potency were made by measuring a dose-ratio at one arbitrary response level (20 mmHg).

Effect of pregnancy and parturition

In eight non-pregnant rabbits, in oestrus or anoestrus, the mean dose (±s.e.) of prostaglandin E_2 required to produce a 20 mmHg fall in diastolic blood pressure was 240 ± 74 ng by the intra-arterial route and 2.51 ± 0.54 μg by the intravenous route. The mean dose-ratio was 14.8 ± 2.6.

In pregnant (day 12) or pseudopregnant (day 12) rabbits, the dose-ratios were significantly higher (25.3 ± 2.4 and 29.0 ± 5.8 respectively, Table 1). By days 22-28 of pregnancy the dose-ratio had risen further to 68.8 ± 3.4. These findings are consistent with there being an increase in the rate of inactivation of prostaglandin E_2 in the lungs during pregnancy and pseudopregnancy.

One of our aims was to measure the inactivation of prostaglandin E_2 in the lung during the process of parturition, which usually occurred on day 32. However, as the process of littering could start at any time on this day and was often over within 30 min, this was impractical. Therefore, experiments were performed on day 32 either before any young were born, or immediately after the full litter had been delivered. In the former group, anaesthesia did not prevent the rabbits from attempting to litter, and while this was occurring the blood pressure showed wide fluctuations which were impossible to distinguish from the depressor responses to the prostaglandin. Thus, in order to carry out the experiments at this stage, it was necessary to delay parturition and hence stabilize the blood pressure. This was

Table 1 Comparison of intravenous and intra-arterial doses of prostaglandin E_2 (PGE_2) causing a 20 mmHg fall in diastolic blood pressure before, during and after pregnancy

Condition	*No. of expts*	*Dose of PGE_2 (mean ± s.e.)*		*Dose-ratio, i.v./i.a.*	
		i.v. (μg)	*i.a. (ng)*	*(Mean ± s.e.)*	*Significance†*
Non-pregnant	8	2.51 ± 0.54	240 ± 74	14.8 ± 2.6	
Pseudopregnant					
day 12	7	3.22 ± 0.61	115 ± 17	29.0 ± 5.8	$P < 0.05$
Pregnant					
day 12	5	4.57 ± 0.53	198 ± 49	25.3 ± 2.4	$P < 0.02$
days 22-28	5	11.59 ± 0.81	171 ± 17	68.8 ± 3.4	$P < 0.001$
days 31-32:					
starting to litter*	4	16.33 ± 5.02	198 ± 27	89.9 ± 34.6	$P < 0.01$
immediately after littering	2	13.05 (17.8, 8.3)‡	152 (139, 165)‡	89.2 (128.0, 50.4)‡	
Post-partum					
1 day	5	6.21 ± 1.94	142 ± 27	46.3 ± 11.1	$P < 0.01$
2-3 days	3	3.69 ± 1.14	123 ± 32	29.0 ± 1.8	$P < 0.02$

* Given indomethacin (10 mg, i.v.) to stop parturition. ‡ Individual values.
† Significance, compared with the non-pregnant group by Student's t test.

achieved within 1 h by an intravenous injection of 10 mg indomethacin, a substance known to delay parturition in the rat (Aiken, 1972; Chester, Dukes, Slater & Walpole, 1972) and in primates (Novy, Cook & Manaugh, 1974). Under these conditions, the dose-ratios were not significantly different from those on days 22-28 of pregnancy (Table 1). It is possible, however, that the dose-ratios we measured following indomethacin administration were artificially low since this drug is a weak inhibitor of prostaglandin catabolism (Flower, 1974).

In two rabbits anaesthetized within approximately 1 h of littering, the blood pressure was stable and no indomethacin was given. The dose-ratios were similar to those before littering (Table 1). Thus there was no evidence that the lung inactivation of prostaglandin E_2 changes appreciably at the time of parturition.

During the first three days post-partum, however, the dose-ratio fell quickly (Table 1), in contrast with its comparatively slow rise during pregnancy. The results indicate that the rate at which the dose-ratio falls may depend on the litter size. For example, rabbits on day 1 post-partum could be classified into two groups, three which had a litter of 6-9 young and dose-ratios between 40-75, and two with litters of 3 young and dose-ratios of 19.3 and 27.3. The reason for this is not clear. Suckling a large litter *per se* did not ensure a high dose-ratio since two rabbits were suckling litters of 7 and 9 young on day 3 post-partum and had comparatively low dose-ratios of 29.7 and 31.8.

The high dose-ratios found in the pregnant and post-partum rabbits were due to a diminished sensitivity to prostaglandin E_2 given intravenously. The sensitivity of these rabbits to the intra-arterial injections did not differ significantly from that of the non-pregnant controls (Figure 1).

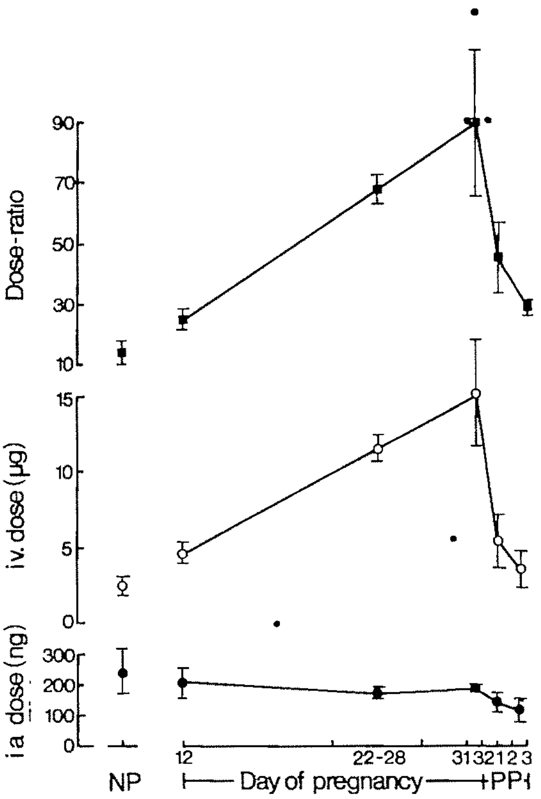

Figure 1 Mean doses (±s.e.) of prostaglandin E_2 required by the intravenous (i.v.) and intra-arterial (i.a.) routes to cause a 20 mmHg fall in diastolic blood pressure in anaesthetized rabbits, either non-pregnant (NP), or on various days of pregnancy or post-partum (PP). The dose-ratio was obtained by dividing the intravenous dose by the intra-arterial dose for each rabbit, and is a measure of the ability of the lung to inactivate prostaglandin E_2. Vertical bars show s.e. mean.

Effect of ovariectomy and of steroid hormones

In three rabbits, ovariectomized 14-17 days before the day of the experiment, the dose-ratios were not significantly different from those in intact non-pregnant controls (Table 2). Thus, this period of deprivation of ovarian hormones had little effect on the inactivation of prostaglandin E_2 by the lung.

Treatment with two hormones, cortisol 25 mg/kg or oestradiol monobenzoate 10 μg/kg, daily for 12 days, had no significant effect on the dose-ratio (Table 2). Treatment with progesterone, 10 mg/kg, or half this dose combined with oestradiol monobenzoate, 5 μg/kg, for 12 days significantly raised the dose-ratio to nearly double that found in rabbits treated with the vehicle (ethyl oleate) alone. These dose-ratios were not dissimilar from those on days 12 of pregnancy and pseudopregnancy. Treatment with progesterone for 26 days raised the dose-ratio almost to the levels found on days 22-28 of pregnancy, although the results were rather variable in the progesterone treated group.

As with the pregnant rabbits, it was the decrease in sensitivity to intravenous prostaglandin E_2 that caused the dose-ratios to be elevated in the progesterone treated rabbits.

In vitro *experiments*

The smooth muscle stimulating activity present in the crude lung enzyme preparations before the addition of any prostaglandin E_2 was equivalent to

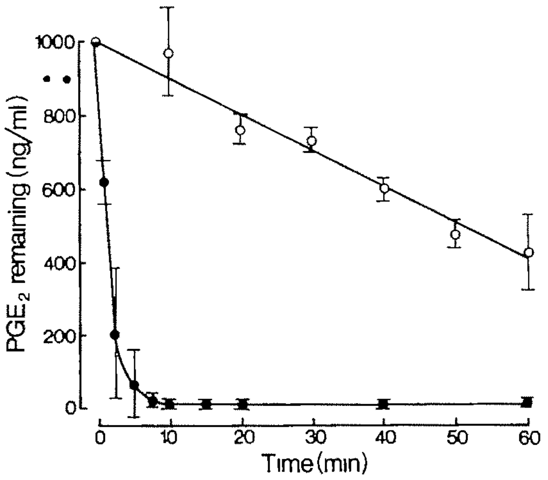

Figure 2 The rate of metabolism of prostaglandin E_2 (PGE_2) in crude enzyme preparations from the lungs of non-pregnant (o) and pregnant (•) rabbits. Each point is the mean value from 3 experiments using enzyme preparations from different rabbits, and refers to the smooth muscle stimulating activity (prostaglandin E_2 equivalents) remaining in the reaction mixture at the times shown. Vertical bars show s.d.

<10 ng prostaglandin E_2/ml in every experiment.

The enzyme preparations from the pregnant rabbits were more potent in inactivating prostaglandin E_2 than those from the non-pregnant rabbits. In the preparations from the pregnant rabbits, the biological activity in the reaction mixture fell from its initial value of 1 µg/ml to 10 ng/ml (prostaglandin E_2 equivalents) in 10-15 minutes. As there was no further loss of biological activity, this residual activity was probably due to the weak biological activity of prostaglandin E_2 metabolites, rather than to unchanged prostaglandin E_2. By contrast, in the preparations from the non-pregnant rabbits, there was still the equivalent of 334-534 ng/ml of prostaglandin E_2 remaining after a 60 min incubation (Figure 2). The half-lives of the prostaglandin in the preparations from pregnant and non-pregnant rabbits were calculated to be approximately 1.5 min and 50 min respectively. The total protein contents of these preparations did not differ significantly, the values being 0.27 ± 0.03 mg/ml and 0 25 ± 0.03 mg/ml respectively (means ± s.e., $n = 3$).

To exclude the possibility that the inactivation of prostaglandin E_2 in the above preparations was caused by blood which had been trapped in the lungs, the stability of the prostaglandin in a 1 in 220 dilution of homogenized blood from pregnant and non-pregnant rabbits was investigated. In neither case was any loss of biological activity detected after incubation for 1 h at 30° C.

Discussion

These experiments show that the capacity of the rabbit lung to inactivate prostaglandin E_2 increases during pregnancy. Most of our evidence was obtained from *in vivo* experiments because we

Table 2 Comparison of intravenous and intra-arterial doses of prostaglandin E_2 (PGE_2) causing a 20 mmHg fall in diastolic blood pressure following ovariectomy or treatment with various steroid hormones

Treatment	*No. of expts*	*Dose of PGE$_2$ (mean ± s.e.)* *i.v. (µg)*	*i.a. (ng)*	*Dose-ratio, i.v./i.a.* *(Mean ± s.e.)*	*Significance†*
Ovariectomy					
14-17 days earlier	3	1.81 ± 0.22	208 ± 84	12.2 ± 4.3	NS
12 days treatment with:					
Ethyl oleate (vehicle)	5	2.76 ± 0.33	152 ± 29	20.1 ± 3.6	
Cortisol 25 mg/kg	3	2.69 ± 0.83	246 ± 115	14.8 ± 7.3	NS
Oestradiol* 10 µg/kg	5	3.37 ± 0.38	152 ± 22	26.2 ± 7.1	NS
Oestradiol* 5 µg/kg with progesterone 5 mg/kg	5	5.10 ± 1.66	149 ± 49	37.5 ± 6.1	$P < 0.05$
Progesterone 10 mg/kg	8	4.60 ± 0.56	142 ± 28	36.7 ± 5.8	$P < 0.05$
26 days treatment with:					
Ethyl oleate (vehicle)	5	1.81 ± 0.29	165 ± 52	13.3 ± 2.7	
Progesterone 10 mg/kg	7	6.61 ± 1 21	121 ± 20	60.6 ± 12.4	$P < 0.02$

* Monobenzoate.

† Significance, compared with ethyl oleate group by Student's t test, except for ovariectomized group which was compared with the 'non-pregnant' group (cf. Table 1). NS, not significant ($P > 0.05$).

wished to maintain as closely as possible the conditions existing in the intact animal. For this reason, too, we gave single injections of prostaglandin E_2 rather than infusions because prostaglandin release from the uterus seems to occur in intermittent short bursts rather than as a continuous output (Thorburn, Cox, Currie, Restall & Schneider, 1972).

A difficulty was encountered in that the dose-response curves measured in these experiments were not parallel, those for the intravenous injections being steeper than those for the intra-arterial injections. It is important, therefore, to consider the likelihood of the depressor responses to intravenous prostaglandin E_2 being due, in part, to an action on sites not immediately available to the prostaglandin injected intra-arterially.

We were able to show that intravenous injections of prostaglandin E_2 did not cause changes in heart rate, or evoke depressor reflexes by an action in the heart or lungs. However, another possible way in which the intravenous injections could have caused exaggerated depressor responses is by reducing the cardiac output, and this might have been brought about in several ways. We avoided the possibility of the prostaglandin acting on capacitance vessels, and thereby reducing venous return, by giving the injections high into the inferior vena cava. Another way in which cardiac output can be reduced is by pulmonary vasoconstriction. The occurrence of this in our experiments seems unlikely, as another prostaglandin of the E series, prostaglandin E_1, decreases pulmonary vascular resistance in the rabbit (Hauge, Lunde & Waaler, 1967). However, the effect of prostaglandin E_2 on this parameter does not appear to have been investigated in the rabbit. Finally, the prostaglandin could have had a direct effect on cardiac contractility. Again, this is unlikely as prostaglandin E_2 has little effect on contractile force in the rabbit isolated heart (Hedqvist, Stjärne & Wennmalm, 1970). *In vivo*, prostaglandin E_2 has no direct effect on cardiac contractility in the cat, and increases it in the dog (Jones, Kane & Ungar, 1974).

There was no evidence that our intra-arterial injections of prostaglandin E_2 caused adrenaline release, and we precluded the possibility that the responses to these injections were modified by a bolus of the prostaglandin passing up the left carotid artery to exert a pressor effect via the central nervous system. Thus, the most likely explanation for the steeper dose-response curves for intravenous prostaglandin E_2 is that the lung inactivation mechanism becomes swamped by high prostaglandin concentrations, so that proportionately more escapes metabolism when large doses are given. It is for this reason that we compared the potencies of intravenous and intra-arterial injections at a fairly low response level, i.e. a fall in blood pressure of 20 mmHg.

The results of the *in vitro* experiments showed that the increased inactivation of prostaglandin E_2 during pregnancy can be attributed directly to an increase in the activity of lung metabolizing enzymes, and not to haemodynamic changes which might occur at this time. The correlation between the results of the *in vitro* and the *in vivo* experiments shows that, although the dose-ratio determined *in vivo* was only an arbitrary measurement of lung inactivation, it does appear to be a valid measurement for comparative purposes.

We have made no attempt to identify the lung prostaglandin-metabolizing enzymes operating in our experiments. However, the enzymes most likely to be involved are prostaglandin 15-hydroxy dehydrogenase and Δ13-prostaglandin reductase (Anggård, 1971). It is not possible to determine from the present results whether the increased lung inactivation of prostaglandin E_2 during pregnancy results from an increased affinity of existing enzymes for the substrate or from an increase in the amount of enzyme present. The total protein content of the crude enzyme preparations from pregnant and non-pregnant rabbit lungs did not differ significantly, but clearly such a measurement would be unlikely to detect changes in the concentrations of specific enzymes which presumably constitute only a small fraction of the total lung cytoplasmic protein. It would appear, however, that there is no general increase in the concentration of lung cytoplasmic enzymes during pregnancy.

Recently, Sun & Armour (1974) have studied in detail the levels of prostaglandin 15-hydroxy dehydrogenase and Δ13-prostaglandin reductase in lungs from pregnant and non-pregnant rabbits. They found a 20-fold increase in dehydrogenase and a 3-fold increase in reductase concentrations at days 28-30 of pregnancy compared with those found in non-pregnant rabbits. This provides a biochemical basis for our observation that the lung is able to inactivate prostaglandin E_2 more efficiently during pregnancy.

It was of course of considerable interest to discover the factor responsible for this change in the lungs of pregnant rabbits. It appeared that the plasma concentration of steroid hormones might be of importance since the dose-ratio was found to be increased in pseudopregnant as well as pregnant rabbits on day 12, and we found progesterone treatment to be effective in increasing the lung inactivation of prostaglandin E_2. However, there cannot be a direct relationship between plasma progesterone levels and lung inactivation, since progesterone levels are at their highest around day 12 of pregnancy and decline subsequently (Challis, Davies & Ryan, 1973a), whereas the lung inactivation shows only a moderate increase at this time, and increases further as pregnancy continues. The possibility that other hormone changes are involved cannot be excluded. For example, the ratio of oestradiol to progesterone increases towards the end of pregnancy (Hilliard, Scaramuzzi, Penardi & Sawyer, 1973), and cortisol levels increase (Mulay, Giannopoulos & Solomon, 1973). However, we found no significant change in lung inactivation when non-pregnant rabbits were treated for 12 days with oestradiol or cortisol.

The slow onset of the increased inactivation during pregnancy compared with its rapid disappearance once the litter was born (Figure 1) is of particular interest. As this slow onset was similar in pregnancy and during progesterone treatment, and since there appears to be no direct correlation between the ability of the lung to inactivate prostaglandin E$_2$ and the level of progesterone in the plasma, the hormone may increase lung inactivation in an indirect manner. This would reconcile our results with the report that progesterone has an inhibitory effect on placental 15-hydroxy-prostaglandin dehydrogenase *in vitro* (Schlegel, Demers, Hildebrandt-Stark, Behrman & Greep, 1974). Plasma prostaglandin F$_{2\alpha}$ levels in the rabbit show a significant increase between days 21 and 30 of pregnancy (Challis, Davies & Ryan, 1973b), and progesterone has been shown to stimulate uterine prostaglandin F$_{2\alpha}$ production in the sheep (Caldwell, Tillson, Brock & Speroff, 1972; Amoroso, Harrison, Heap & Poyser, 1973). Thus, perhaps an increased level of prostaglandins in venous blood is the stimulus which brings about an increased lung inactivation.

Although we have confined our experiments to the rabbit, there is evidence that a similar effect also occurs in the sheep. Oakes, Mofid, Brinkman & Assali (1973) could not obtain significant changes in blood pressure in pregnant sheep with large amounts of prostaglandin E$_2$ injected intravenously, and concluded that the sheep is insensitive to prostaglandins, apparently unaware of the findings of Horton, Main & Thompson (1965) that prostaglandins of the E series have marked effects on blood pressure in non-pregnant sheep. Recently, Horton & Maule Walker (personal communication) have shown that the results of Oakes *et al.* (1973) can be explained partially by an enhanced inactivation of prostaglandin E$_2$ by the sheep lung during pregnancy, and, in addition, have confirmed our observation that this occurs in the rabbit.

Horton & Maule Walker (1975) also attribute some of the insensitivity of the pregnant sheep and rabbit to a decreased sensitivity of the arterial vasculature to prostaglandins. In our experiments, however, the pregnant rabbits were no less sensitive to intra-aortic prostaglandin E$_2$ than the non-pregnant animals, as is clear from Figure 1.

If an enhanced lung inactivation of prostaglandin E$_2$ occurs in pregnant women also, then two points need to be considered. Firstly, although intravenous infusions of prostaglandin E$_2$ are effective in inducing labour (Karim, Hillier, Trussell, Patel & Tamusange, 1970), only a very small proportion of the infused dose would reach the uterus. Thus the human uterus at term might be even more sensitive to prostaglandins than is at present supposed. Secondly, as prostaglandins of the E and F series have been found in the ante-cubital venous blood of women during parturition (Karim, 1968; Hertelendy *et al.*, 1973), assuming that these prostaglandins originate from the uterus, the amounts released into the uterine venous blood must be very large. In such a situation, enhanced lung inactivation would facilitate the removal of prostaglandins from the circulation and could have an important protective function.

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THE EFFECT OF RESERPINE ON HYPOTHALAMO-PITUITARY-ADRENOCORTICAL FUNCTION IN THE RAT

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1 The effect of reserpine on hypothalamo-pituitary-adrenocortical (HPA) function in the rat was investigated by the use of direct and indirect indices of pituitary adrenocorticotrophic activity.

2 Administration of a single dose of the drug induced prolonged hypersecretion of corticotrophin (ACTH).

3 Corticotrophin release in response to the drug no longer occurred after repeated daily injections, indicating that some form of 'adaptation' occurred.

4 The increase in HPA activity normally caused by exposure to cold was prevented by reserpine once 'adaptation' to the drug had been produced.

5 Inhibition of stress-induced ACTH release was due neither to depletion of pituitary stores of the hormone, nor to a corticosteroid feedback effect.

Introduction

Although the effect of reserpine on hypothalamo-pituitary-adrenocortical (HPA) function has been extensively studied, its action on the secretion of corticotrophin (ACTH) is still not fully understood. Some reports indicate that the drug reduces the activity of the HPA system probably by depressing hypothalamic regions which control the output of ACTH (Mason & Brady, 1956; Wells, Briggs & Munson, 1956; Mahfouz & Ezz, 1958), whereas others suggest that it increases the functional activity of the system (Gaunt, Renzi, Antonchak, Miller & Gilman, 1954; Egdahl, Richards & Hume, 1956; Harwood & Mason, 1957; Saffran & Vogt, 1960; Montanari & Stockham, 1962).

The experiments described here were carried out in an attempt to clarify some of the discrepancies in the literature, by the use of direct estimates of blood and pituitary ACTH for the assessment of pituitary-adrenocorticotrophic activity in addition to the indirect indices which have been almost exclusively employed in the past.

Methods

Animals

Male Sprague-Dawley rats (Fisons Pharmaceuticals Ltd.) weighing 150-250 g were used. The animals were housed two per cage in a temperature-controlled room (22° C) for at least 5 days before the start of any treatment. Food (Diet 41B, Lane-Petter & Dyer, 1952) and water were always available *ad libitum.*

Drugs

Reserpine (Halewood Chemicals Ltd.) was dissolved in 1% glacial acetic acid in deionized water. The pH of the solution was adjusted to 4 with 4 M NaOH and the concentration to 2.5 mg reserpine/ml. Less concentrated solutions were prepared by dilution of the stock solution with vehicle (i.e. 1% glacial acetic acid in deionized water, adjusted to pH 4). The solution was injected intraperitoneally in volumes of 1 ml/kg body weight, in doses of either 1.25 or 2.5 mg reserpine/kg body weight. Controls received 1 ml/kg of the vehicle by the same route. All injections were given in the morning between 09 h 00 min and 10 h 00 minute. In contrast to the vehicle-treated animals, the reserpine-treated rats had diarrhoea for the first 3-4 days of treatment and exhibited ptosis, slight ataxia, pilo-erection and loss of body weight.

Cold stress

Cold stress was applied by placing the cages, each containing two rats, in a large, ventilated refrigerator maintained at 4° C.

Blood samples

Blood for plasma corticosterone determination only was collected under ether anaesthesia from the abdominal aorta, into a heparinized 5 ml syringe. The samples were collected within 2 min of removing the animals from their cages, centrifuged at 3,000-3,500 rev/min within 1 h of collection and the plasma stored at −10° C. The plasma corticosterone concentration was estimated fluorimetrically by the method of Zenker & Bernstein (1958).

Blood for the estimation of plasma ACTH concentration was collected from the trunk following rapid decapitation with a pair of large, sharp, scissors. The blood was strained through heparinized glass wool, collected in chilled heparinized plastic tubes and centrifuged immediately at 3,000-3,500 rev/min for not more than 5 minutes. Volumes (0.3 ml) of plasma were placed in chilled, stoppered plastic tubes which were then stored on solid CO_2 in the deep freeze. The plasma ACTH concentration was estimated by the redox bioassay method (Chayen, Loveridge & Daly, 1972) in which ACTH-induced changes in the stainable reducing activity in the zona reticularis of the guinea-pig adrenal are measured by scanning and integrating microdensitometry.

Adrenal glands

Adrenal glands were removed immediately after withdrawal of blood from the abdominal aorta, freed from peri-adrenal fat and connective tissue and weighed on a torsion balance. Each gland was transferred to a test-tube containing 12 ml of 4% (w/v) trichloroacetic acid solution and carefully ground with the aid of sand and a glass rod. Adrenal ascorbic acid concentrations were determined by the method of Roe & Kuether (1943).

Pituitary glands

After decapitation the top of the cranium was cut away and the cerebral hemispheres were carefully lifted back to expose the pituitary gland. The gland was removed, weighed on a torsion balance and homogenized in 1 ml cold 0.1 M HCl. The homogenate was placed in a chilled plastic tube, each tube containing two pituitaries from a given group of animals, and stored overnight in a refrigerator. The following day the tubes were transferred to the deep freeze and stored on solid CO_2. Pituitary ACTH content was estimated by the redox bioassay method (Chayen *et al.*, 1972).

Results

The effect of reserpine on adrenal ascorbic acid, plasma corticosterone and plasma ACTH concentration, and pituitary ACTH content

Adrenal ascorbic acid concentrations, adrenal weights and plasma corticosterone and ACTH concentrations after single intraperitoneal injections of reserpine are shown in Table 1 and Figure 1. In the vehicle-treated group the plasma ACTH concentration rose significantly ($P < 0.01$), reached a maximum value between 2.5 and 10 min and returned to the basal level within 40 min of the injection. In the reserpine-treated group the plasma ACTH concentration reached a similar

Table 1 Adrenal ascorbic acid concentrations, adrenal weights, plasma corticosterone concentrations and plasma ACTH concentrations in rats 24 h after single intraperitoneal injections of reserpine or vehicle

Treatment	*Adrenal weight (mg)*	*Adrenal ascorbic acid concentration (mg/100 g)*	*Plasma corticosterone concentration (µg/100 ml)*	*Plasma ACTH concentration (mu/100 ml)*
None	31.5 ± 0.56 (19)	425.5 ± 5.5 (56)	8.2 ± 0.3 (48)	0.56 ± 0.05 (20)
Vehicle (6)	32.6 ± 0.85	438.9 ± 15.5	8.05 ± 0.9	0.50 ± 0.1
Reserpine 1.25 mg/kg (6)	37.8 ± 2.8*	367.8 ± 25.9**	22.7 ± 2.1*	2.8 ± 0.4*
Reserpine 2.5 mg/kg (6)	36.8 ± 1.4*	363.3 ± 17.2**	20.0 ± 2.1*	3.6 ± 0.7*

Values are means ± s.e. mean.
Significantly different from control values *$P < 0.001$, **$P < 0.01$.
Numbers of animals used in parentheses.

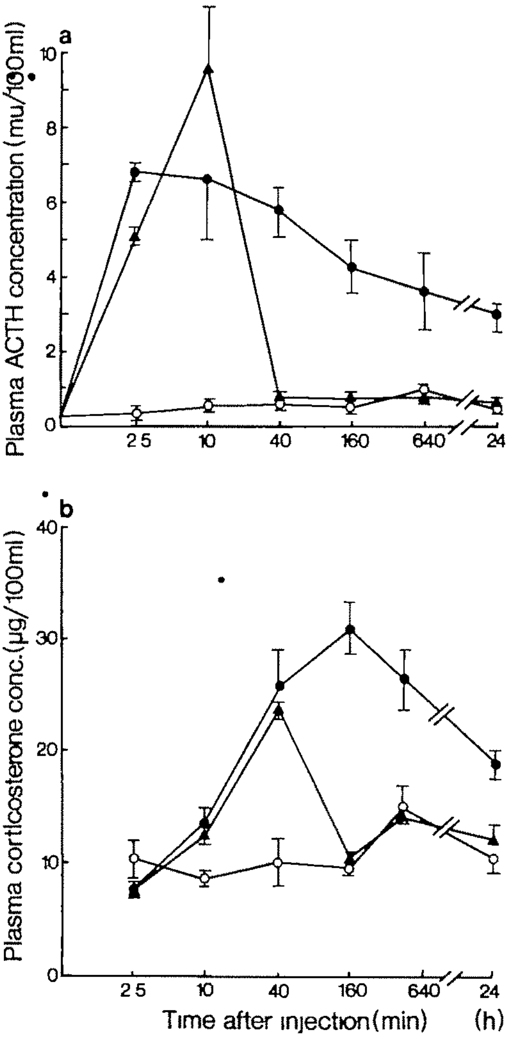

Figure 1 Plasma ACTH and corticosterone concentrations in rats at various time intervals after a single intraperitoneal injection of reserpine, 2.5 mg/kg (●) or vehicle, 1 ml/kg (▲); untreated controls (○). All injections were given at 09 h 00 minute. Each point represents the mean of at least 6 determinations. Vertical bars show s.e. mean.

peak between 2.5 and 10 min after the injection but then declined very gradually and remained significantly ($P < 0.001$) above the basal level for at least 24 hours. The plasma corticosterone concentration showed a similar response. In the vehicle-treated group it reached a peak in 40 min and then fell sharply to the control value. The initial increase in plasma corticosterone was similar in the animals treated with reserpine but instead of falling to the control level after 40 min, it continued to rise, reaching a maximum in 160 minutes. The plasma corticosterone concentration subsequently decreased gradually but remained significantly ($P < 0.001$) greater than the control value, suggesting that a single intraperitoneal injection of reserpine induces hypersecretion of ACTH for at least 24 hours.

The effects of injections of reserpine repeated at daily intervals are shown in Figure 2 and Tables 2 & 3. The first injection of the drug caused adrenal ascorbic acid depletion and an increase in the plasma corticosterone concentration 1 h after injection. This response was no longer evident 1 h

Table 2 Plasma ACTH concentration 2.5 min after the last of repeated daily injections of reserpine or vehicle

Treatment	*No. of daily injections*	*Plasma ACTH (mu/100 ml)*
–	–	0.56 ± 0.05 (20)
Vehicle (1 ml/kg)	1	5.6 ± 0.3* (6)
Vehicle (1 ml/kg)	3	4.5 ± 1.1* (6)
Vehicle (1 ml/kg)	5	5.8 ± 1.0* (6)
Vehicle (1 ml/kg)	7	3.1 ± 0.9* (8)
Reserpine (2.5 mg/kg)	1	6.8 ± 1.5* (7)
Reserpine (2.5 mg/kg)	3	1.1 ± 0.5* (6)
Reserpine (2.5 mg/kg)	5	0.96 ± 0.4† (6)
Reserpine (2.5 mg/kg)	7	0.56 ± 0.1 (6)

Values are means ± s.e. mean.
Significantly different from control values *$P < 0.001$, †$P < 0.01$.
Numbers of animals indicated in parentheses.

Table 3 Pituitary ACTH content in rats 'adapted' to reserpine and subjected to cold stress (5 min at 4°C) immediately after the last reserpine injection

Treatment	*ACTH mu per pituitary*	
	Before stress	*After stress*
–	115.6 (6)	120 (6)
Reserpine, 2.5 mg/kg once daily for 7 days	66.7 (8)	60.3 (8)
Vehicle once daily for 7 days	62.0 (6)	61.0 (6)

Each value is the mean of three samples, each containing two pooled pituitaries. Numbers of animals used in parentheses.

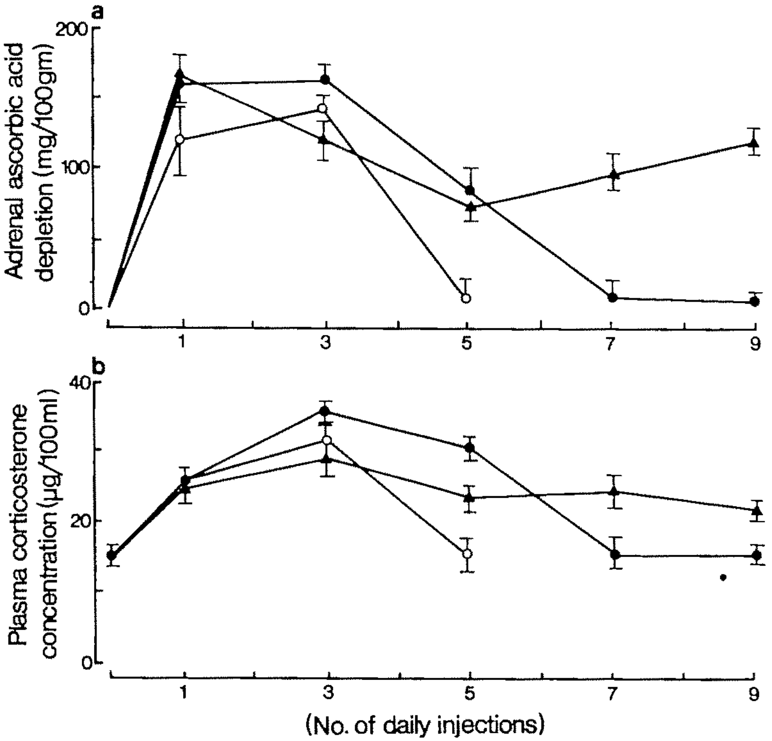

Figure 2 (a) Adrenal ascorbic acid depletion and (b) plasma corticosterone concentration after repeated daily intraperitoneal injections of reserpine, 1.25 mg/kg (●), 25 mg/kg (○) and vehicle (▲). All observations were made 1 h after the final injection. Each point represents the mean of at least 6 determinations. Vertical bars show s.e. mean.

after the 5th and 7th injection of 2.5 and 1.25 mg reserpine/kg respectively (Figure 2). Injection of the vehicle alone also caused adrenal ascorbic acid depletion and an increase in the plasma corticosterone concentration but the responses persisted even after the 9th injection. The results indicate that some form of 'adaptation' occurs in response to repeated daily injections of the drug but not to the vehicle alone. Direct estimates of plasma ACTH after repeated injections of reserpine confirm that 'adaptation' to the drug occurs and the plasma ACTH concentration was unchanged 2.5 min after the 7th injection (Table 2). The pituitary ACTH content (Table 3) after repeated injections of reserpine was similar to that in the corresponding vehicle-treated group.

The effect of cold stress on adrenal ascorbic acid, plasma corticosterone and plasma ACTH concentration, and pituitary ACTH content after treatment with reserpine

Exposure to cold (4° C for 1 h) caused a decrease in the adrenal ascorbic acid concentration and a marked increase in the plasma corticosterone concentration in untreated control rats. In rats 'adapted' to reserpine (5 daily injections of 2.5 mg/kg) and in vehicle-treated controls, exposure to cold for 1 h immediately after the final injection caused no change in adrenal ascorbic acid or plasma corticosterone concentration (Figure 3). However, the adrenal ascorbic acid was already depleted and the plasma corticosterone raised at the time of stress in the vehicle-treated group but not in the reserpine-treated rats. Twenty-four hours after the last injection, when adrenal ascorbic acid and plasma corticosterone concentrations were normal, cold stress caused a significant fall in the concentration of adrenal ascorbic acid and a rise in that of plasma corticosterone ($P < 0.01$ and $P < 0.001$ respectively) in the vehicle-treated control group. In the corresponding group of reserpine-treated rats exposure to cold caused a significant ($P < 0.001$) rise in the concentration of plasma corticosterone but no concomitant adrenal ascorbic acid depletion. Hence, if adrenal ascorbic acid depletion is taken as the index of

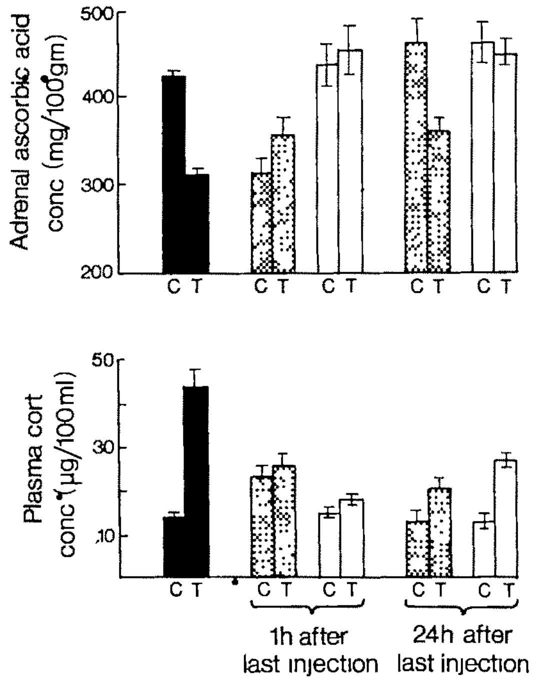

Figure 3 Adrenal ascorbic acid and plasma corticosterone concentrations in rats adapted to reserpine (2.5 mg/kg), subjected to cold stress and killed immediately afterwards. Solid columns, untreated controls, dotted columns, five daily injections of vehicle only, open columns, five daily injections of reserpine (2.5 mg/kg), C, before stress; T, 1 h at 4° C. Each column represents the mean of at least 6 determinations. Vertical bars show s.e mean.

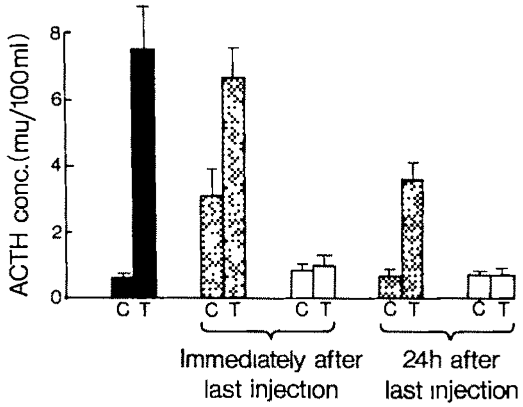

Figure 4 Plasma ACTH concentrations in rats subjected to cold stress after 'adaptation' to reserpine (2.5 mg/kg). Rats were killed immediately after cold exposure. Solid columns, untreated controls, dotted columns, seven daily injections of vehicle only, open columns, seven daily injections of reserpine (2.5 mg/kg); C, before stress; T, 5 min at 4° C. Each column represents the mean of at least 6 determinations. Vertical bars show s.e. mean.

pituitary-adrenocorticotrophic activity, inhibition of ACTH release is apparent 1 h and 24 h after the final injection of reserpine but if changes in plasma corticosterone concentration are used it is apparent only 1 h later.

Blood and pituitary ACTH were estimated in rats 'adapted' to reserpine after exposure to cold for 5 min (Figure 4 and Table 3). The stress caused a marked rise in the concentration of ACTH in the blood of untreated control animals with no concomitant change in the pituitary content of the hormone. Reserpine-treated rats exposed to cold either immediately or 24 h after the 7th injection showed no significant change in the concentration of ACTH in the plasma or its content in the pituitary gland, in contrast to the corresponding vehicle-treated control animals in which a significant increase ($P < 0.05$) in the plasma ACTH concentration occurred but no change in pituitary ACTH content.

Discussion

These results indicate that reserpine is capable of blocking the stress-induced release of ACTH, although it may powerfully stimulate secretion of the hormone when administered acutely. A persistent and prolonged hypersecretion of ACTH following a single injection of reserpine has often been observed (Wells *et al.*, 1956; Kitay, Holub & Jailer, 1959; Saffran & Vogt, 1960; Maickel, Westermann & Brodie, 1961; Eechaute, Lacroix, Leusen & Bouckaert, 1962; Montanari & Stockham, 1962; Bhattacharya & Marks, 1969). This response to a single injection of the alkaloid is reduced and ultimately disappears if the injections are repeated daily. Animals 'adapted' to the effects of the drug in this way no longer release ACTH in response to cold stress. Our results have confirmed the findings of Wells *et al.* (1956), Kitay *et al.* (1959) and Maickel *et al.* (1961) that the stress-induced release of corticotrophin may be inhibited by reserpine. They also explain the failure of Eechaute *et al.* (1962) and Montanari & Stockham (1962) to demonstrate the phenomenon because of their lack of recognition of the need for their experimental animals to become 'adapted' to reserpine-treatment before the application of stressful stimuli.

Some of our data using adrenal ascorbic acid changes as the index of ACTH release are not in complete agreement with the data obtained using plasma corticosterone changes. A dissociation between these two indices has been reported previously (Slusher, 1958; Eskin & Mikhailova, 1968) and some of the discrepancies in the literature may also be explained on this basis. The obvious limitations of indirect indices for the assessment of pituitary-adrenocorticotrophic activity were overcome in the present work by use of the sensitive, precise and specific redox bioassay for ACTH (Chayen *et al.*, 1972) and the ability of reserpine to block stress-induced ACTH secretion was confirmed.

The results obtained with the vehicle-treated controls are interesting. Indirect indices of pituitary-adrenocorticotrophic activity suggest that the vehicle is capable of suppressing the stress-induced release of the hormone. Such inhibition of ACTH secretion was confirmed by direct estimates of plasma ACTH, which showed that injection of the vehicle alone acts as a profound stress, stimulating ACTH release, and that repeated exposure to such a stress results in a subsequent decrease in the response to cold stress. Similar observations have been reported previously (Stark, Fachet & Mihàly, 1963; Jones & Stockham, 1966) and emphasize the need for proper controls. This is a factor which has been neglected by some investigators (Mahfouz & Ezz, 1958; Kitay *et al.*, 1959).

The precise mechanism whereby reserpine exerts its effect on HPA function is uncertain. Failure to release ACTH in response to stress has been attributed to depletion of pituitary stores of the hormone (Kitay *et al.*, 1959; Maickel *et al.*, 1961). However, although it has been shown that a single injection of reserpine causes a marked fall in the pituitary ACTH content (Saffran & Vogt, 1960) our data indicate clearly that inhibition of ACTH release after 'adaptation' to reserpine occurs when pituitary stores of the hormone are not different from the control value. In any event, it is unlikely that the pituitary ACTH content provides a reflection of the functional capacity of the gland (Sayers & Cheng, 1949; Marks & Vernikos-Danellis, 1963, Vernikos-Danellis, 1963; Hodges & Jones, 1964). Moreover, the suppression of ACTH release was not due to a corticosteroid feedback mechanism as has been suggested by Giuliani, Motta & Martini (1966), because it occurred when reserpine-treatment caused no change in the concentration of corticosterone in the plasma. Probably its effects on HPA function are associated with changes in hypothalamic catecholamines or 5-hydroxytryptamine, as was suggested by Westermann, Maickel & Brodie (1962), Bhattacharya & Marks (1969) and Marks, Hall & Bhattacharya (1970).

Our results indicate that reserpine inhibits the functional activity of the HPA system and partly explain how the use of different, relatively insensitive indices of ACTH secretion and the neglect of proper controls have led to many discrepancies in the literature and to widely different interpretations of the experimental data.

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EFFECT OF FENFLURAMINE ON 5-HYDROXYTRYPTAMINE UPTAKE AND RELEASE BY RAT BLOOD PLATELETS

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1 (+)-Fenfluramine reduces the central stores of 5-hydroxytryptamine (5-HT) by a poorly understood mechanism.

2 Rat blood platelets have been used in this study as a simple model for serotoninergic nerve endings.

3 (+)-Fenfluramine shows a dual effect: it inhibits the uptake of [^{14}C]-5-HT by platelets and it releases newly absorbed [^{14}C]-5-HT from platelets.

4 The inhibition of [^{14}C]-5-HT uptake induced by (+)-fenfluramine appears very rapidly, is concentration-dependent and seems not to be competitive. (+)-Fenfluramine is ten times less effective than chloroimipramine but ten times more effective than (+)-amphetamine; (+)-fenfluramine is more active than its (−)-isomer or its metabolite norfenfluramine ((+)- or (−)-form).

5 The release of [^{14}C]-5-HT from platelets induced by (+)-fenfluramine is concentration-dependent but increases with increased incubation time. Both chloroimipramine and (+)-amphetamine are in comparison very poor release inducers; (+)-fenfluramine is more active than its (−)-isomer or its metabolites.

6 The effect on [^{14}C]-5-HT uptake exerted by (+)-fenfluramine and chloroimipramine *in vitro* could not be observed *in vivo*.

7 The observed effect of fenfluramine on the uptake and release of 5-HT may explain the lowering action of fenfluramine on the brain 5-HT level, an effect considered of importance for the anorectic effect of this drug.

Introduction

Fenfluramine is a drug capable of suppressing food intake in animals (Le Douarec, Schmitt & Laubie, 1966) and in man (Munro, Seaton & Duncan, 1966), which unlike amphetamine, is devoid of central stimulant activity (Bizzi, Bonaccorsi, Jespersen, Jori & Garattini, 1970; Le Douarec & Neveu, 1970). A characteristic effect of fenfluramine on brain neurotransmitters is a long lasting, dose-dependent lowering of brain 5-hydroxytryptamine (5-HT) (Duhault & Verdavainne, 1967; Opitz, 1967; Costa, Groppetti & Revuelta, 1971) and 5-hydroxyindoleacetic acid (5-HIAA) (Opitz, 1967; Garattini, 1973). Since relatively little information is available on the mechanism by which fenfluramine reduces the central stores of 5-HT (Morgan, Cattabeni & Costa, 1972; Ghezzi, Samanin, Bernasconi, Tognoni, Gerna & Garattini, 1973) it was decided to investigate the effect of fenfluramine on the uptake or the release of 5-HT.

The study was carried out on blood platelets, considered by several authors as a simple model for nerve endings (Paasonen, 1965; Pletscher, 1968; Tuomisto, 1974). The selected animal species was the rat as most studies of the effects of fenfluramine on 5-HT have been carried out in this species; in addition, rat platelets are unable to metabolize 5-HT (Paasonen, 1965).

Methods

Preparation of platelet-rich plasma (PRP) and platelet-poor plasma (PPP)

Blood was obtained from male Charles River rats (weight 170-200 g), anaesthetized with ether, by intracardiac puncture with a 10 ml plastic syringe containing 1 ml 3.13% trisodium citrate for 9 ml of blood. Throughout the experiments only disposable plastic material was used. Blood was centrifuged at 400 *g* for 15 min and the PRP was pipetted off. Platelets were counted by phase microscopy and adjusted to about 600,000/μl by appropriate dilutions with PPP from the same animal. PPP was obtained by further centrifugation of blood at 3,000 *g* for 20 minutes.

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Preparation of plasma-free platelets (PFP)

PRP (10 ml) was gel filtered on a Sepharose 2 B column (2.0 x 20.0 cm) according to the method described by Tangen, Berman & Marfey (1971). The number of PFP was adjusted to about 600,000/μl by appropriate dilution with the elation buffer.

Uptake of [^{14}C]-5-hydroxytryptamine by platelets

In vitro *experiments* PRP (1.9 ml) or PFP was first preincubated at 37° C with 0.1 ml of 0.9% w/v NaCl solution (saline) or drug solution, then with either 0.05 μg, 0.1 μg or 0.2 μg [^{14}C]-5-HT, corresponding to 0.007 μCi/ml, 0.014 μCi/ml and 0.028 μCi/ml respectively. The mixtures were gently shaken during the incubation period. Incubation was stopped by cooling the tubes in a melting ice-bath; afterwards the samples were centrifuged at 4° C at 3,000 *g* for 20 min and the supernatant carefully collected.

Radioactivity (ct/min) was counted in duplicated samples for 1 min in a liquid scintillation counter (Beckman LS 250) after addition of 0.2 ml uncentrifuged PRP or PFP or supernatant to 15 ml of a dioxane-naphthalene scintillation mixture. As rat blood platelets do not contain monoamine oxidase (Paasonen, 1965), it was assumed that radioactivity was a measure of 5-HT uptake. Uptake of [^{14}C]-5-HT was calculated as described by David & Hérion (1972). Preliminary experiments showed no difference in radioactivity counting when a given amount of [^{14}C]-5-HT was added to equal volumes of distilled water, PRP or PPP, both in presence and in absence of (+)-fenfluramine. At any time, the sum of radioactivity counted in the supernatant and in the platelet pellet (the latter carefully resuspended in saline) corresponded to the amount (97-98%) of the added radioactivity.

In vivo *experiments* Rats were injected intraperitoneally with one of the following drugs: (+)-fenfluramine (either 15 or 30 mg/kg body wt) and chloroimipramine (10 or 20 mg/kg body wt.). The animals were killed 15, 30, 60 and 120 min after drug administration.

In other experiments rats received (+)-fenfluramine (5 mg/kg, i.p.) or chloroimipramine (10 mg/kg, i.p.) twice a day for 6 days; 12 h after the last injection the animals were killed and blood collected as usual. Uptake of [^{14}C]-5-HT by platelets was studied in PRP as described above.

Release of [^{14}C]-5-hydroxytryptamine by platelets

Samples of 1.9 ml PRP were incubated with 0.2 μg [^{14}C]-5-HT (corresponding to 0.028 μCi/ml) for 15 min; to induce release of the accumulated 5-HT, 0.1 ml of different drug solutions was added to the samples which were further incubated at 37° C for 15, 60 or 120 minutes. The radioactivity in the supernatant was counted as described above. Release induced by the different drugs was then calculated from the difference between samples incubated with and without addition of the drugs under the same experimental conditions (David & Hérion, 1972).

Evaluation of other platelet functions

Changes in platelet shape and aggregation were studied at 37° C in a Born-Michal MK IV Aggregation and Shape-Change Monitor (Pharmacological Research, England) connected to a two-channel chart recorder (Servoscribe 2, type RE 520, Smith's Industries Ltd., England) (Born, 1970). A sample (0.7 ml) of PRP was preincubated for 15 min with 0.1 ml saline with or without (+)-fenfluramine (10^{-4} M); then 0.2 ml of one of the following substances was added: adenosine-5'-diphosphate, disodium salt (ADP) (Sigma, USA) (at concentrations ranging between 2.10^{-4} and 10^{-7} M), undiluted Thrombofax (a commercial cephalin prepared as an ether extract of acetone-dried bovine brain by Ortho, U.S.A.) or collagen (acid soluble, Hormon; Germany) 5-20 μg/ml.

Drugs

5-Hydroxytryptamine-[3'-^{14}C] creatinine sulphate (55 mCi/mmol) was obtained from Radiochemical Centre, Amersham, dissolved in 70% ethanol and stored at −20° C. 5-Hydroxytryptamine creatinine sulphate (Farmitalia, Italy), (+)-fenfluramine hydrochloride, (−)-fenfluramine hydrochloride (+)-norfenfluramine hydrochloride and (−)-norfenfluramine hydrochloride (Servier, France), chloroimipramine (Ciba Geigy, Switzerland) and (+)-amphetamine sulphate (Recordati, Italy) were dissolved in saline. Final concentrations are indicated throughout.

Results

In vitro experiments

Uptake of [^{14}C]-5-hydroxytryptamine The percent uptake of [^{14}C]-5-HT by platelets was very rapid; it reached its maximum within 1 min, was unrelated to the concentration of [^{14}C]-5-HT used and did not increase after 1 h of incubation (Table 1). When platelets were first preincubated for either one or 15 min with (+)-fenfluramine (2.5×10^{-5} M) and subsequently with [^{14}C]-5-HT, a marked inhibition of the uptake of the radioactive amine was observed; this uptake was almost completely inhibited during the first minute after the addition of the labelled material, then slowly increased and reached a plateau after 15 min (Table 1). Similar results were obtained when platelets were preincubated for 15 min with chloroimipramine (Table 1). The inhibition of platelet uptake of [^{14}C]-5-HT was linearly related to the concentration of the drugs used and was not competitive; compared to (+)-fenfluramine, chloroimipramine was ten times more effective; in contrast, (+)-amphetamine had ten times less inhibitory effect than (+)-fenfluramine (Figure 1). Prolongation of the preincubation time to 4 h only slightly increased the inhibitory effect of (+)-fenfluramine.

The inhibitory activity of (+)-fenfluramine on [^{14}C]-5-HT uptake was compared with that of (+)-norfenfluramine, (−)-fenfluramine and (−)-nor-fenfluramine both in PRP and PFP; these compounds were used at the same final concentration as (+)-fenfluramine (2.5×10^{-5} M) which gave 50% inhibition. The results shown in Table 2, indicate that in PRP (+)-fenfluramine was the most effective substance, whereas in PFP no difference could be observed between (+)-fenfluramine and (+)-norfenfluramine; in both systems, the (+)-compounds were more active than the (−)-compounds.

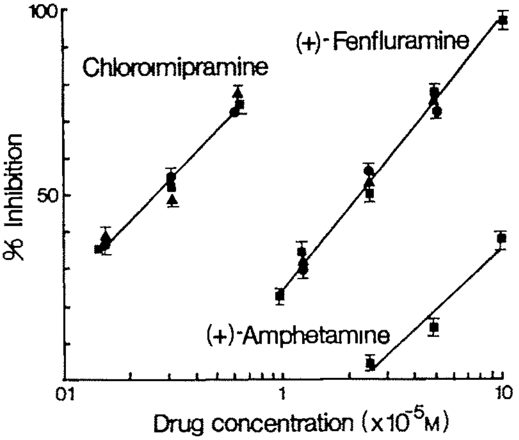

Figure 1 Dose-response curves for the inhibitory activity of chloroimipramine, (+)-fenfluramine and (+)-amphetamine on [^{14}C]-5-hydroxytryptamine (5-HT) uptake by rat platelets. Each point represents the mean of four duplicated experiments. Vertical bars show s.e.mean. Drugs were preincubated with platelet rich plasma for 15 min before an additional incubation period of 15 min with [^{14}C]-5-HT (0.2 µg (■); 0.1 µg (▲) or 0.05 µg (●)).

Release of [^{14}C]-5-hydroxytryptamine Incubation of labelled platelets with (+)-fenfluramine for 15 min provoked a release of [^{14}C]-5-HT which was concentration-dependent. When the incubation was prolonged up to 2 h, the release of [^{14}C]-5-HT was greatly increased. Both (+)-norfenfluramine and (−)-fenfluramine were less effective than (+)-fenfluramine in inducing [^{14}C]-5-HT release whereas (−)-norfenfluramine was almost ineffective. Chloroimipramine even at a concentration (5×10^{-5} M) which completely inhibited the uptake of [^{14}C]-5-HT, provoked a small release of radioactivity. Similarly (+)-amphetamine was poorly active in releasing 5-HT from platelets (Table 3).

Table 1 Kinetics of the uptake of [^{14}C]-5-hydroxytryptamine (5-HT) by rat blood platelets. Effects of (+)-fenfluramine and chloroimipramine.

Time (min) after addition of 0.05 µg [^{14}C]-5-HT	% uptake of [^{14}C]-5-HT Control	(+)-Fenfluramine (2.5×10^{-5} M)		Chloroimipramine (3.1×10^{-6} M)
1	95.0 ± 2.3	8.2 ± 2.6*	4.6 ± 1.0**	2.0 ± 1.1**
2	—	17.0 ± 1.0	9.7 ± 0.7	4.5 ± 2.6
3	—	16.2 ± 2.4	15.5 ± 1.5	12.5 ± 1.8
5	—	24.7 ± 3.1	20.0 ± 1.9	17.0 ± 1.9
10	—	35.7 ± 4.6	34.7 ± 1.2	33.5 ± 3.6
15	95.7 ± 1.0	45.5 ± 5.2	43.1 ± 6.0	42.2 ± 3.3

Results are mean ± s.e.mean of four duplicated experiments.
* Drug added to PRP 1 min before [^{14}C]-5-HT. ** Drug added to PRP 15 min before [^{14}C]-5-HT.

Table 2 Comparison of the inhibitory effect of (+)-fenfluramine, (−)-fenfluramine and their metabolites on [^{14}C]-5-hydroxytryptamine (5-HT) uptake by rat platelets, suspended either in plasma (PRP) or in a plasma-free medium (PFP).

Drug	*% Inhibition*	
(2.5 x 10^{-5} M)	*PRP*	*PFP*
(+)-Fenfluramine	52.0 ± 2.8	40.1 ± 2.9
(+)-Norfenfluramine	27.0 ± 1.5	39.0 ± 3.1
(−)-Fenfluramine	26.3 ± 2.1	16.4 ± 0.7
(−)-Norfenfluramine	3.1 ± 0.2	4.3 ± 0.3

Results are mean ± s.e.mean of four duplicated experiments.

Table 3 Percent release of platelet-bound [^{14}C]-5-hydroxytryptamine (5-HT) induced by incubation of PRP with different drugs for 2 hours

Drugs (5 x 10^{-5} M)	*% Release*
Chloroimipramine	14.3 ± 0.3
(+)-Amphetamine	16.7 ± 1.4
(+)-Fenfluramine	52.7 ± 3.0
(+)-Norfenfluramine	41.7 ± 0.3
(−)-Fenfluramine	39.7 ± 0.3
(−)-Norfenfluramine	6.3 ± 0.3
Saline	<2.0

Results are mean ± s.e.mean of four duplicated experiments.

In vivo *experiments*

The influence of (+)-fenfluramine given intraperitoneally to rats on the subsequent *in vitro* uptake of [^{14}C]-5-HT by platelets was studied after both acute and chronic treatments and compared with the results of analogous experiments performed with chloroimipramine; an appreciable inhibition of the uptake (23.8% ± 4.1 and 14.0% ± 7.9 respectively) was only found 15 min after a single injection of either drug. No inhibition was observed at 120 min or after a repeated treatment (6 days) with either drug.

Other platelet functions

In vitro, shape change and platelet aggregation induced by ADP, collagen and Thrombofax were not modified by (+)-fenfluramine. This drug even at high concentrations (10^{-4} M) neither provoked platelet aggregation *in vitro* (up to 4 h incubation) nor influenced the platelet count after acute or repeated *in vivo* treatments.

Discussion

The experiments described here indicate that fenfluramine interferes with the storage and/or the transfer of 5-HT in rat blood platelets, used in this study as a simple model of serotoninergic nerve terminals. In this *in vitro* model fenfluramine has a dual effect: it inhibits 5-HT uptake and it induces release of 5-HT. No other effect of fenfluramine on platelet number and function could be detected either *in vitro* or *in vivo*, indicating that the drug does not cause nonspecific damage to the platelet membrane.

The inhibition of 5-HT uptake appears very rapidly and is concentration-dependent (fenfluramine 1-10 x 10^{-5} M). The effect of fenfluramine is not inhibited by increasing the concentration of 5-HT four-fold, suggesting that the inhibition is not of a competitive nature. Fenfluramine is about ten times less active than a classic inhibitor of 5-HT uptake such as chloroimipramine, but it is at least ten times more active than its analogue (+)-amphetamine. (+)-Fenfluramine appears to be more active than its (−)-isomer or its metabolite, norfenfluramine ((−)- or (+)-form). This finding further supports the suggestion that fenfluramine acts on the serotoninergic system *per se* rather than through the formation of norfenfluramine (Morgan *et al.*, 1972; Garattini, Buczko, Jori & Samanin, 1974). In a plasma-free medium, however, no differences were observed between (+)-fenfluramine and (+)-norfenfluramine; this could be explained by a stronger binding of norfenfluramine to plasma proteins; however the role of plasma proteins in our system requires further investigation.

As far as the release is concerned, fenfluramine, at concentrations comparable to those which inhibit uptake, is effective in decreasing the levels of labelled 5-HT in platelets. The effect of fenfluramine on 5-HT release is concentration-dependent and increases with the time of incubation. As was seen in the uptake experiments, (+)-fenfluramine is also more active than its (−)-isomer or its metabolite in inducing [^{14}C]-5-HT release from platelets. In contrast, chloroimipramine, which is much more effective than (+)-fenfluramine as an inhibitor of uptake, is a very poor release inducer. Amphetamine is also much less effective than (+)-fenfluramine in releasing 5-HT from platelets. It is remarkable that the effect on 5-HT uptake exerted by fenfluramine and chloroimipramine *in vitro* could not be observed *in vivo* during acute or repeated treatments. This may be due to the fact that both drugs penetrate very rapidly into tissues and so only very low concentrations are found in plasma (Ghezzi *et al*, 1973; Bizzi, Tacconi, Tognoni, Morselli & Garattini, unpublished observations).

The observed effect of fenfluramine on the uptake and on the release of 5-HT may help to explain the reduction in brain 5-HT levels caused by fenfluramine (Duhault & Verdavainne, 1967; Opitz, 1967; Costa *et al.*, 1971), an effect considered of importance for the anorectic effect of this drug (Jespersen & Scheel-Krüger, 1970; Samanin, Ghezzi, Valzelli & Garattini, 1972). In fact the inhibitory effect on uptake together with the releasing action may also explain the reduction in brain 5-HIAA levels caused by fenfluramine (Duhault & Verdavainne, 1967), since it is believed that 5-HT is chiefly deaminated intraneuronally (Blaschko & Levine, 1966). The inhibition of 5-HT uptake is not sufficient alone to lower brain 5-HT or 5-HIAA (Carlsson, Corrodi, Fuxe & Hökfelt, 1969; Ghezzi *et al.*, 1973), unless there is a concomitant release of 5-HT, as for instance during electrical stimulation of the midbrain raphe (Samanin, Ghezzi & Garattini, 1972). The possibility that the inhibition of 5-HT uptake and the release of 5-HT may be important factors in the reduction of brain 5-HT produced by fenfluamine is, in any case, reinforced by other experimental studies showing that this anorectic drug does not inhibit tryptophan hydroxylase, monoamine oxidase (Morgan, Lõfstrandh & Costa, 1972) or 5-HT synthesis (Costa *et al.*, 1971). On the contrary, there may be an increase in 5-HT synthesis which may depend on an increased level of tryptophan in the brain after administration of fenfluramine (Costa *et al.*, 1971). It may be added that recently Fuxe (1974) working with brain slices *in vitro* has also observed a dual effect of fenfluramine on uptake and release of 5-HT.

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INTERACTION OF HISTAMINE H_1- AND H_2-RECEPTOR ANTAGONISTS WITH HISTAMINE UPTAKE AND METABOLISM BY GUINEA-PIG ISOLATED ATRIUM AND MOUSE NEOPLASTIC MAST CELLS *in vitro*

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1 Burimamide, metiamide, chlorpheniramine, triprolidine and cocaine, were tested as inhibitors of histamine uptake and metabolism in the guinea-pig atrium and in mouse neoplastic mast cells.

2 Cocaine did not affect the uptake and metabolism of histamine, either in the atrium or in the mast cells. All the antihistamines tested blocked the uptake and metabolism of histamine in both preparations. The order of potency was burimamide > chlorpheniramine > triprolidine > metiamide in the atrium; and burimamide > metiamide > triprolidine > chlorpheniramine, in the mast cells.

3 Comparison of the present results with the antihistamine activity of these blocking agents suggests that no correlation exists between the receptor blocking activity and the ability of these substances to act as inhibitors of histamine uptake and metabolism.

Introduction

Traditional antihistamines (H_1-receptor blocking agents; Ash & Schild, 1966) as well as the more recent H_2-receptor blocking agents (burimamide and metiamide: Black, Duncan, Durant, Ganellin & Parsons, 1972; Black, Duncan, Emmet, Ganellin, Hesselbo, Parsons & Willie, 1973) are thought to inhibit the response to histamine by a primary action at the receptor site. However, it is possible that some antihistamines might antagonize the effects of histamine by preventing the uptake of both exogenous and locally released histamine. In fact, a variety of antihistamines potentiate the arterial pressor response and the positive chronotropic action of noradrenaline in the rat heart, by inhibiting, like cocaine, the amine uptake at the neuronal membrane (Isaac & Goth, 1965; Johnson & Kahn, 1966; Isaac & Goth, 1967).

The purpose of the present study was to determine whether H_1- and H_2-receptor antagonists prevent the uptake of histamine in guinea-pig heart and mouse neoplastic mast cells and if so, to see whether this effect is related to their histamine receptor blocking properties.

The experiments were carried out on guinea-pig atria and mouse neoplastic mast cells, in an attempt to differentiate between the effects of triprolidine, chlorpheniramine, burimamide and metiamide in preparations in which the accumulation of histamine is mainly due to uptake (mast cells: Moroni, Buiatti & Mannaioni, 1972), or to uptake and metabolism (isolated atrium: Mannaioni & Moroni, 1973).

Methods

Experiments on isolated atria

The interactions of antihistamines with histamine uptake and metabolism were studied in isolated left atria of guinea-pigs prepared according to the procedure described by Furchgott, Kirpekar, Rieker & Schwab (1963). Each atrium was placed under a tension of 1 g in a 5 ml muscle chamber and perfused with Tyrode solution at 30° C, through which a mixture of 97% O_2 and 3% CO_2 was bubbled. The composition of the perfusion fluid (mM) was: NaCl 136.9; KCl 2.7; $CaCl_2$ 1.8 (titrated); $MgCl_2$ 1.0; NaH_2PO_4 0.4; $NaHCO_3$ 11.9; glucose 5.6. The pH of the solution was 7.45. The preparations were stimulated electrically (0.5 ms duration; twice threshold voltage; 2 Hz) and contractions were recorded with a Battaglia-Rangoni polygraph. After an equilibration period, the atria were perfused either with [^{14}C]-histamine or with [^{14}C]-histamine plus various concentrations of drugs over a period of 30 min, and then washed for 10 min to remove extracellular histamine (Mannaioni & Moroni, 1973).

The chronotropic response to noradrenaline in the presence of cocaine and antihistamines was measured on isolated, spontaneously beating atria of the guinea-pig. The atrial beats were recorded by attaching the atria to an isometric Grass transducer under a tension of 1 g, connected to a Battaglia-Rangoni polygraph.

Having established the resting atrial rate, (±)-noradrenaline was added to the bath at 2 min intervals in final concentrations ranging from 10^{-10} to 10^{-5} M. After this procedure, the atrium was repeatedly washed until the control rate was re-established. One of the test compounds was then added to the bath, followed by the cumulative graded concentrations of the amine.

Experiments on mast cells

The experiments were carried out on a clone of murine neoplastic mast cells originating from the Furth mastocytoma (HC subline: Mannaioni, Fischer & Giarman, 1968). The clone was maintained as an ascitic tumor in LAF$_1$ mice (Jackson Memorial Laboratory). The histamine content of the cells continuously grown in mice was fairly constant (Mannaioni, 1970). Periodic microscopic examination revealed a quite uniform cell population, accounted for almost completely by mast cells (95-98%).

Treatment with drugs was carried out as follows: cells from mice bearing the ascitic tumour were removed (usually 5-7 days after the inoculum) by aspiration of peritoneal fluid, collected by centrifugation and washed with 5 ml of ice-cold Tyrode solution. The cells were harvested and resuspended in a medium containing [^{14}C]-histamine, and [^{14}C]-histamine plus different concentrations of antihistamines. After incubation for 1 h (gas phase: air; pH 7.45) a cell count was taken; the cells were harvested, washed three times and extracted. All samples were run in duplicate. All the drugs were dissolved in Tyrode at pH 7.45 and added to the cell suspension in a ratio never exceeding 1 : 10 ml.

Extraction and analyses

Atria and mast cells were homogenized in 0.4 N perchloric acid and suitable aliquots were taken for the assay of total radioactivity, histamine and methylhistamine.

In the atria, total radioactivity was measured in the perchloric acid extract. The [^{14}C]-histamine assay was carried out according to the isotope dilution method of Schayer (1968). The [^{14}C]-methyl-histamine was assayed by the method of Snyder, Axelrod & Bauer (1964). Aliquots of the perchloric acid extracts of the atria were extracted into alkaline chloroform. After washing the chloroform layer, methyl-histamine was transferred to acid and counted. The difference between the total radioactivity and that referring to [^{14}C]-histamine plus [^{14}C]-methyl-histamine accounted for the sum of the other metabolites.

In mast cells, assay by the isotope dilution method showed that [^{14}C]-histamine accounted for all the radioactivity, so that in further experiments this procedure was considered no longer necessary, and the amount of radioactivity found in perchloric extracts was directly referred to as [^{14}C]-histamine.

The endogenous histamine content of the atria and mast cells was determined by means of a biological assay carried out on the guinea-pig ileum as described by Giotti, Guidotti, Mannaioni & Zilletti (1966).

Drugs

Histamine-[ring 2-^{14}C] dihydrochloride, specific activity 54 mCi/mmol, was purchased from the Radiochemical Centre, Amersham.

Burimamide and metiamide were obtained from Dr J.W. Black, Smith Kline & French Laboratories. Other substances used were: triprolidine hydrochloride, (Wellcome); chlorpheniramine maleate, (Schering); cocaine hydrochloride, (Carlo Erba); (±)-noradrenaline hydrochloride, (Calbiochem); histamine dihydrochloride, (Calbiochem).

Results

Chronotropic responses to noradrenaline in the presence of cocaine and antihistamines

Figure 1 shows that triprolidine did not significantly shift the dose-response curve to noradrenaline, while chlorpheniramine increased the positive chronotropic effect of noradrenaline, especially at the lowest concentrations. Higher concentrations of antihistamines directly depressed the atrial rate, while lower concentrations were ineffective.

Burimamide and metiamide failed to shift the dose-response curve to noradrenaline, even at high concentrations, while cocaine, at a concentration of 10^{-6} M shifts this curve upwards and to the left (Figure 2). None of the antihistamines studied modified the atrial rate at the concentrations used.

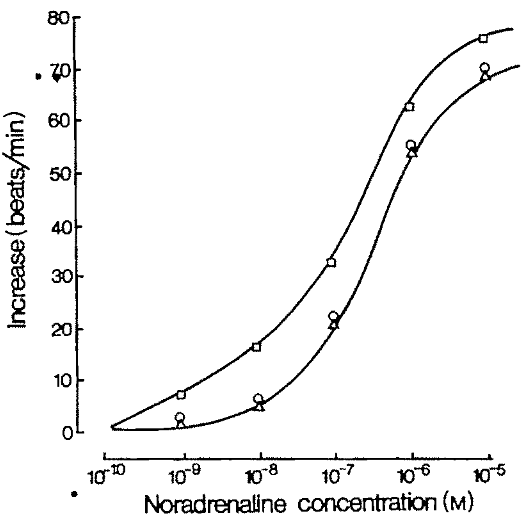

Figure 1 Effect of triprolidine and chlorpheniramine on the chronotropic action of noradrenaline in guinea-pig atria. Control (○); triprolidine 10^{-6} M (△); chlorpheniramine 10^{-6} M (□). The control curve is the mean of 10 experiments; the curves for triprolidine and chlorpheniramine are both the mean of 5 experiments. For each point the s.e. mean fell between 1.7 and 7.8 beats/minute.

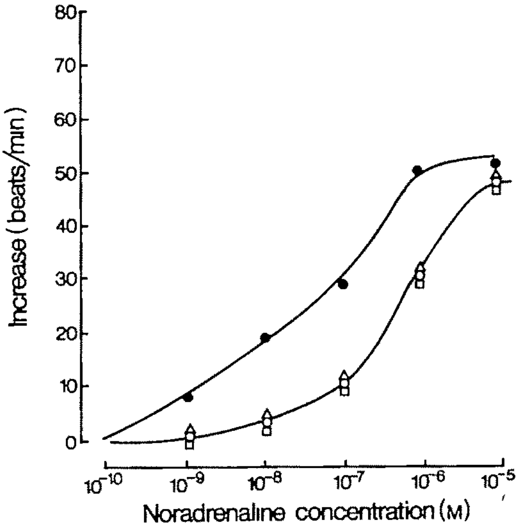

Figure 2 Effect of cocaine, burimamide and metiamide on the chronotropic action of noradrenaline in guinea-pig atria. Control (○); cocaine 10^{-6} M (●); burimamide 3×10^{-4} M (△); metiamide 10^{-4} M (□). The control curve is the mean of 15 experiments; the other curves are each the mean result of 5 experiments. For each point the s.e. mean fell between 1.3 and 7.1 beats/minute.

Effects of drugs on histamine uptake and metabolism in the atria

At the highest concentrations used, none of the drugs studied (triprolidine; chlorpheniramine; burimamide; cocaine) altered the endogenous histamine content.

The H_1-receptor blocking agents, triprolidine and chlorpheniramine, prevented the accumulation of total radioactivity, as well as the formation of methylhistamine, in a dose-dependent fashion. However, at the same concentrations they did not significantly block the uptake of the unchanged histamine (Table 1). By measuring the drug concentration required to produce a 50% inhibition of histamine methylation (IC_{50}), it was possible to show that chlorpheniramine was more effective than triprolidine in affecting this process (Table 3).

The H_2-receptor blocking agents, burimamide and metiamide, behave similarly to triprolidine and chlorpheniramine in inhibiting the accumulation of total radioactivity and the formation of methyl-histamine, without impairing the uptake of unchanged histamine. However, the IC_{50} clearly revealed that burimamide is the most active compound, while metiamide blocked the uptake of radioactivity and the histamine methylation only at high concentrations.

It is interesting to note that cocaine, at the concentration used (10^{-6} M) was devoid of any significant action on the accumulation and metabolism of histamine (Table 1).

Effect of drugs on histamine uptake by mast cells

Cell viability and endogenous histamine content were unchanged by exposure of cells to drug concentrations up to 10^{-4} M.

Cocaine was completely ineffective in blocking histamine uptake at all concentrations used. Chlorpheniramine showed a clear inhibition of the uptake process only at high concentrations. Triprolidine and metiamide were almost equiactive, although they blocked histamine uptake more effectively than chlorpheniramine. Burimamide was clearly the most active compound, showing a significant inhibition of histamine uptake even at low concentrations (Table 2).

The IC_{50} of the four antihistamines were, for burimamide 1.8 μM, for metiamide 28 μM, for triprolidine 20 μM and for chlorpheniramine 60 μM.

Table 1 Effect of antihistamines and cocaine on the uptake and metabolism of [^{14}C]-histamine by guinea-pig isolated atria

Treatment	*Concentration of drug* (M)	*Total radioactivity*	*Methyl-histamine*	*Histamine*
			(d/min x 10^{-3}) per g	
Control	—	170 ± 7	43 ± 3	7.8 ± 0.7 (12)
Triprolidine	3.10^{-7}	152 ± 20	40 ± 2	7.5 ± 1 (4)
	10^{-6}	133 ± 18*	34 ± 5	6.1 ± 1.3 (4)
	10^{-5}	125 ± 4*	28 ± 4*	6.2 ± 1.2 (4)
	10^{-4}	72 ± 0.7*	7.9 ± 1*	6.2 ± 1.5 (4)
Chlorpheniramine	3.10^{-7}	180 ± 20	46 ± 3	7.6 ± 1 (4)
	10^{-6}	133 ± 2*	28 ± 3*	6.2 ± 0.9 (4)
	10^{-5}	123 ± 7*	17 ± 2*	5.2 ± 1.2 (4)
	10^{-4}	71 ± 6*	8 ± 0.9*	5.2 ± 1.7 (4)
Burimamide	3.10^{-7}	124 ± 7*	24 ± 3*	9.1 ± 2 (4)
	10^{-6}	71 ± 3*	14 ± 4*	8.7 ± 2 (4)
	10^{-5}	36 ± 5*	5 ± 0.5*	8 3 ± 15 (4)
	10^{-4}	26 ± 4*	5 ± 0.5*	9.6 ± 4 (4)
Metiamide	3.10^{-7}	166 ± 17	38 ± 2	8.1 ± 0.7 (4)
	10^{-6}	140 ± 9*	42 ± 3	8.1 ± 0.7 (4)
	10^{-5}	109 ± 7*	37 ± 2	8 ± 1.2 (4)
	10^{-4}	53 ± 7*	8 ± 0.9*	7.3 ± 3 (4)
Cocaine	10^{-6}	141 ± 7	47 ± 9	8 ± 2 (4)

Atria were incubated with [^{14}C]-histamine (100 ng/ml) for 30 min and washed for 10 min before extraction and analysis.
Number of experiments in parentheses. Values are means with s.e.
* Statistically significant difference from controls ($P < 0.01$) according to the analysis of variance.

Table 2 Effect of antihistamines and cocaine on the uptake of [^{14}C]-histamine by murine neoplastic mast cells

Concentration of drugs (M)	*Burimamide*	*Metiamide*	*Triprolidine*	*Chlorpheniramine*	*Cocaine*
		Histamine (d/min x 10^{-6}) cells			
0 (controls)	5308 ± 460 (4)	6087 ± 580 (4)	3121 ± 315 (4)	3705 ± 416 (4)	3608 ± 300 (4)
10^{-7}	—	6656 ± 800 (4)	—	3797 ± 633 (4)	— —
10^{-6}	—	6520 ± 1000 (4)	2633 ± 350 (4)*	4157 ± 589 (4)	—
3 x 10^{-6}	2047 ± 70 (4)*	6330 ± 980 (4)	—	3974 ± 564 (4)	3888 ± 50 (4)
10^{-5}	1066 ± 63 (4)*	4600 ± 648 (4)*	2043 ± 137 (4)*	3853 ± 662 (4)	4188 ± 350 (4)
3 x 10^{-5}	494 ± 26 (4)*	2891 ± 200 (4)*	935 ± 170 (4)*	3439 ± 873 (4)	4216 ± 425 (4)
10^{-4}	215 ± 14 (4)*	1255 ± 79 (4)*	294 ± 32 (4)*	805 ± 132 (4)*	3218 ± 400 (4)

The cells were incubated with [^{14}C]-histamine (100 ng/ml) for 60 min, washed three times and extracted.
Number of experiments in parentheses. Values are means with s.e.
* Statistically significant difference from controls ($P < 0.01$).

Discussion

The present experiments have shown that in the guinea-pig atrium all the antihistamines studied inhibit the accumulation of total radioactivity and the methylation of histamine, without affecting the uptake of the unchanged amine; in mouse mast cells, they simply block the uptake of histamine.

These effects are unrelated to the actions shown by the same drugs in shifting the cumulative dose-response to noradrenaline. Burimamide, metiamide and triprolidine do not potentiate noradrenaline, although they significantly inhibit the accumulation of labelling and the formation of methyl-histamine. Moreover, cocaine itself, at concentrations capable of increasing the positive chronotropic effect of exogenous noradrenaline failed to modify the uptake and metabolism of histamine, both in the atrium and in the mast cells.

It is possible that in the guinea-pig atrium the decreased formation of methyl-histamine produced by the antihistamines would lead to an equilibrium between the intraceullular and extracellular concentrations of histamine, thus blocking a further accumulation of label in the cells.

In mast cells, which are incapable of metabolizing histamine (Moroni *et al.*, 1972) it is possible that a competition for histamine binding sites by antihistamines would lead to a steady state between intracellular and extracellular histamine, thus blocking the uptake of histamine into the cells.

In our experiments, the inhibition of histamine methylation is shared by H_1- and H_2-receptor antagonists, although they differ in relative potency (burimamide > chlorpheniramine > triprolidine > metiamide). Classical antihistamines have been shown to inhibit histamine methyltransferase from different sources *in vitro* (Netter & Bodenschanz, 1967; Taylor & Snyder, 1972; Barth, Niemeyer & Lorenz, 1973a); the same holds true for H_2-antagonists (Schayer & Reilly, 1973; Barth, Niemeyer & Lorenz, 1973b).

However, no correlation exists between the ability of antihistamines to inhibit histamine methyltransferase activity and their antihistaminic potency, since the concentrations needed to inhibit histamine methylation differ from those required to block cardiac H_1- and H_2-receptors (Table 3).

In conclusion it is evident that some antihistamines are capable of blocking the uptake and metabolism of histamine. The reported antihistaminic potency of these compounds does not correlate with their ability to block histamine uptake and metabolism as determined in these experiments. These effects therefore do not appear to be related to the antihistaminic property *per se* but rather to other properties, which may be tentatively identified as an inhibition of histamine methylating enzymes, or competition at the binding sites for histamine uptake.

Table 3 Comparison of the relative potencies of the antihistamines with their effectiveness in blocking histamine-methylation

	K_D (μM)*	IC_{50} (μM)°
Triprolidine	–	14
Chlorpheniramine	–	5
Burimamide	7.8	0.47
Metiamide	0.92	30

* Receptor drug dissociation constants; data from Black & Spencer (1973). Positive chronotropic action of histamine on guinea-pig atria.

° IC_{50} Concentration of drug which caused 50% inhibition of histamine methylation in the guinea-pig atria. It was evaluated by plotting on probability log-paper the mean percentage inhibition against the molar concentration of the drug.

We are most grateful to Dr J.W. Black of Smith, Kline & French Laboratories for gifts of burimamide and metiamide. This investigation was supported by grants from the Consiglio Nazionale delle Ricerche, Rome and from the Consiglio di Amministrazione, University of Florence, Italy.

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ANTAGONISM OF ADENOSINE 5'-TRIPHOSPHATE-INDUCED RELAXATION BY 2-2'-PYRIDYLISATOGEN IN THE TAENIA OF GUINEA-PIG CAECUM

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1 2-2'Pyridylisatogen tosylate (PIT) (>2.5 µM) relaxed the guinea-pig isolated taenia caeci by an unknown mechanism.

2 With higher concentrations of PIT (>12.5 µM) subsequent applications of adenosine 5'-triphosphate (ATP) (2-600 µM) revealed a blockade of the ATP receptors. The antagonism was characterized by a delayed onset of action (>10 min incubation with 50 µM PIT) and eventually became irreversible (>50 µM PIT for >30minutes). The antagonism was specific for ATP, was not competitive, and was not dependent upon the relaxant effect.

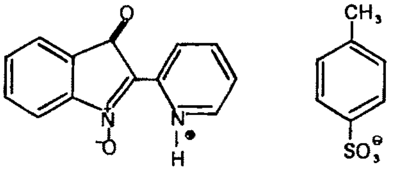
3 The presence of either acetylcholine (0.05-1.0 µM) or carbachol (0.05-1.0 µM) increased the antagonistic effect of PIT (50 µM) approximately five-fold.

4 Following prolonged exposure, PIT (50 µM for 90 min) did not block the inhibitory effects of field stimulation (2 Hz, 10 s) of the taenia caeci in the presence of hyoscine (0.33 µM). These results do not support the purinergic nerve hypothesis.

Introduction

Atropine does not completely block the effects of parasympathetic nerve stimulation to many organs; in the gut, for example, contractions are often converted to relaxations (McSwiney & Robson, 1929; McSwiney, 1931; Paton & Vane, 1963; Campbell, 1966; Burnstock, Campbell, Satchell & Smythe, 1970; Furness, 1970; Beani, Bianchi & Crema, 1971; Burnstock, 1972). In many situations, adenosine 5'-triphosphate (ATP) mimics these atropine-resistant effects, a finding which led Burnstock and his colleagues to propose that ATP or a related purine nucleotide is the chemical transmitter released when the nerves are stimulated. Much evidence supports this claim (see Burnstock *et al.*, 1970; Burnstock, 1972), but the lack of a specific ATP receptor antagonist has led to the use of very high concentrations of some drugs to provide support for the hypothesis. Phentolamine, tolazoline, antazoline and yohimbine have been used by Satchell, Burnstock & Dann (1973) in an attempt to show a similar blockade of exogenous ATP and field stimulation on the guinea-pig taenia caeci. All these drugs have a diversity of pharmacological actions (Boyd, Chang & Rand, 1960; Goodman & Gilman, 1970).

During routine testing of compounds synthesized in our department we discovered that the water soluble tosylate salt of 2-2'pyridylisatogen (PIT) blocked the inhibitory action of ATP on isolated taenia caeci (Hooper, Spedding, Sweetman & Weetman, 1974). In the present paper we report the actions of this compound on the isolated taenia caeci of the guinea-pig, stimulated with drugs and via the nerves.



2-2'Pyridylisatogen tosylate (PIT)

Methods

Taenia caeci preparations were obtained from female guinea-pigs in the weight range 250-600 g. The preparations were arranged in 10 ml or 100 ml organ baths filled with McEwen's (1956) solution maintained at 35 ± 1°C and oxygenated with 95% O_2 and 5% CO_2. After an equilibration period of 30 min, responses were recorded isotonically on a smoked drum (magnification 1 : 4, load 1.5 g).

In some experiments, preparations were stimulated transmurally by rectangular pulses from an SRI 6051 stimulator, by means of electrodes similar to those described by Birmingham &

Wilson (1963). Stimulation characteristics were as follows: pulse duration 0.5 ms; train duration 10 s; interval between pulse trains 2 or 4 min; 150 V setting on the stimulator was used.

Some preparations were stimulated via the perivascular nerves (Burnstock, Campbell & Rand, 1966). Shielded platinum electrodes (Palmer, type H87) were used, the stimulation characteristics being as described above except that the train duration was 15 s and the voltage supramaximal.

Cumulative concentration-response curves were obtained for noradrenaline and ATP at 20 min intervals (Van Rossum, 1963). Each dose of agonist was allowed to produce its full effect (5-13 s contact) before the concentration in the bath was increased. Preparations with low tone, i.e. those which did not contract to 25% of their relaxed length, were discarded.

Drugs

PIT was synthesized by the method of Patterson & Wibberley (1965) and the tosylate salt prepared. Other drugs used were: acetylcholine chloride, (−)-ascorbic acid, carbachol chloride and histamine acid phosphate (B.D.H.); guanethidine sulphate (Ismelin–CIBA); hyoscine hydrobromide B.P., quinidine sulphate B.P. (Mawson and Proctor); imidazole (Sigma); (−)-isoprenaline sulphate (K and K Labs. Inc.); (−)-noradrenaline bitartrate (Koch-Light); phentolamine mesylate B.P. (CIBA); prostaglandin E_2 (Upjohn); tetrodotoxin (Sankyo); adenosine 5′-triphosphate (B.D.H. or Sigma). The catecholamines were protected from oxidation by the inclusion of approximately 100 μg/ml of (−)-ascorbic acid in each dilution. Prostaglandin E_2 was dissolved by the method of Bennett & Posner (1971). Solutions of ATP and imidazole were adjusted to pH 7.4 by the addition of 1.0 M NaOH and 1.0 M HCl respectively. The composition of the McEwen's (1956) solution was as follows (mM): NaCl 130; KCl 5.6; $CaCl_2$ 2.2; $NaHCO_3$ 25; NaH_2PO_4 1.2; glucose 11.1 and sucrose 13.2.

Statistical analyses and other procedures

When complete concentration-response or frequency-response curves were obtained, responses were calculated as percentage maximum relaxation. Relaxations in the presence of an antagonist were expressed as percentage of the control relaxations. Dose-ratios were calculated as the number of times the agonist concentration had to be increased to maintain the 50% maximal response in the presence of the antagonist, the 50% maximal response being calculated from the individual curves. The slopes of the concentration-response curves were calculated as the ratios of the concentrations producing 80% and 20% maximal effects (Stephenson, 1956). Student's t-test was used for comparisons; homogeneity of variance was checked by a variance ratio test (Snedecor & Cochran, 1967).

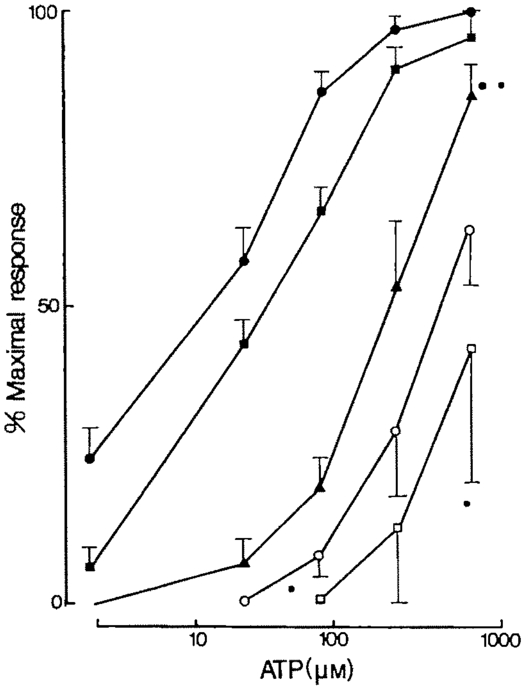


Figure 1 The influence of the duration of exposure on the antagonistic effect of 2-2′pyridylisatogen (PIT) to adenosine 5′-triphosphate (ATP) on taenia caeci. Cumulative concentration response curves to ATP were obtained in the absence (●) and in the presence of PIT (25 μM) after 30 (■), 60 (▲), 90 (○) and 120 (□) min contact. Each point is the mean of eight determinations; vertical bars show s.e.mean. Carbachol (0.05-1.0 μM) was used to restore the tone of the preparations to within 20% of the original level.

Results

Antagonism of ATP-induced relaxations by PIT

ATP (2-600 μM) produced a concentration-dependent, rapid relaxation of the guinea-pig isolated taenia caeci. The relaxation was probably a direct effect on the smooth muscle because tetrodotoxin (0.33 μM) did not modify the responses (n = 6). Concentration-response curves, constructed from experiments in which the tissue

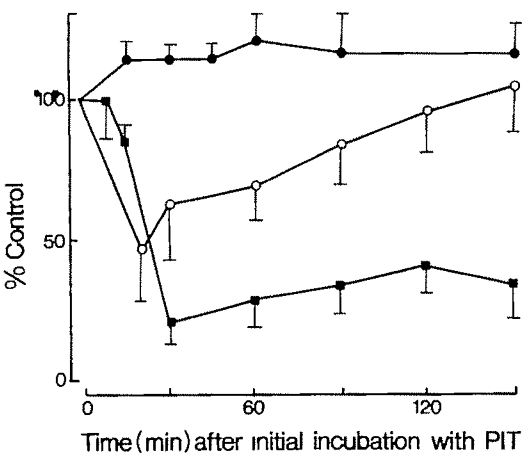


Figure 2 The effect of washing on the antagonism of submaximal responses to adenosine 5'-triphosphate (ATP) by 2-2'pyridylisatogen (PIT) on the taenia caeci. The curves are as follows: control ATP (10 μM) responses (●), ATP after exposure to PIT (50 μM) for 20 (○) or 30 (■) min, starting at time zero. The values represent the mean of five determinations. Vertical bars show s.e.mean. Carbachol (0.05-0.5 μM) was used to restore the tone of the preparations to within 20% of the original length in the initial stages, but was not required for later determinations. Preparations were washed with McEwen's solution (1 litre/hour). Note the delayed onset of the antagonism.

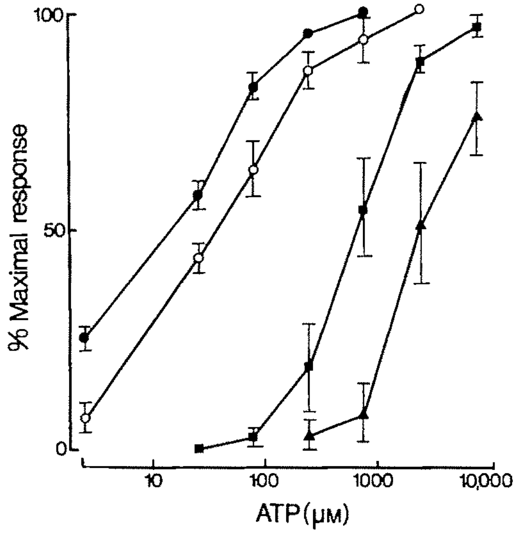


Figure 3 The effect of different concentrations of 2-2'pyridylisatogen (PIT) on the cumulative concentration-response curves to adenosine 5'-triphosphate (ATP). The curves are as follows: control (●), 25 (○), 50 (■) and 75 (▲) μM PIT. The antagonist was incubated with the tissue for 30 min and carbachol (0.05-1.0 μM) was used to restore tone. The values represent the mean of eight determinations. Vertical bars show s.e mean.

was dosed cumulatively, showed that there was no tachyphylaxis to ATP, provided that the initial curve was disregarded (5 curves over 100 min) ($n = 5$). In all subsequent experiments the effect of the first cumulative exposure of the tissue to ATP was not measured.

PIT exerted two actions on the taenia caeci; first, low concentrations (threshold at 2.5 μM) slowly relaxed the preparation. The second effect, a blockade of the inhibitory action of ATP, was seen with higher concentrations of PIT (25 μM or more). In order to detect the antagonism, the taenia, which had been relaxed by the PIT, had to be re-contracted. Acetylcholine (0.05-1.0 μM) and carbachol (0.1-1.0 μM) were routinely used for this purpose (see below). Only preparations that could be restored to within 20% of their length during the control part of the experiment were used.

Both the magnitude and specificity of the ATP antagonism depended upon the duration of incubation of the tissue with PIT. Figure 1 shows how the antagonism of ATP-induced relaxations increased with prolonged exposure of the taenia caeci to PIT 25 μM. If equilibrium between the antagonist and the ATP receptors had been established in these experiments, the curves would not have been progressively displaced to the right; so it can be concluded that PIT did not reach equilibrium within 120 minutes.

The combination of PIT with the ATP receptors on the taenia caeci was reversible in the initial stages, but later became irreversible. Figure 2 depicts a series of five experiments in which the submaximal responses of the taenia caeci to single doses of ATP were investigated after either 20 or 30 min exposure to 50 μM PIT. It can be seen that the response to ATP was restored to the control level following 20 changes of bathing solution when the incubation had been for 20 min, but there was little restoration of sensitivity to ATP following a 30 min exposure. With lower concentrations of PIT (12.5 μM) the antagonism could be reversed after a longer exposure, but this too eventually became irreversible ($n = 2$). A concentration-dependent delay in the onset of the block characterized all the experiments.

The antagonism of ATP-induced relaxations of the taenia caeci by PIT was concentration-dependent (Figure 3). In a series of eight experiments the concentration-response curves for ATP were displaced to the right to an extent determined by the concentration of the PIT. The slopes of these curves were not significantly different from the control ($P > 0.05$), except at the highest concentration tested (75 µM). The data in Figure 3 were analysed by the method of Arunlakshana & Schild (1959), and yielded an apparent pA_2 of 4.8, the slope being 2.6.

The effect of the presence of spasmogenic agents on the antagonism

Table 1 shows the activities of PIT when different spasmogens were used to restore the tone of the taenia caeci. The antagonism of ATP by PIT was significantly greater when the cholinomimetics were used to restore tone to the taenia caeci than occurred with histamine, prostaglandin E_2, or in those rare preparations that did not appreciably relax in response to PIT (3 out of 70). If acetylcholine (0.05-1.0 µM) or carbachol (0.05-1.0 µM) were used to recontract the tissue after PIT, small regular spontaneous contractions which were similar to those seen before the addition of drugs were obtained. In contrast with this histamine (0.2-2.5 µM) induced much larger spontaneous contractions (see Figure 5).

A second method was used to investigate the antagonism of ATP by PIT in the absence of exogenous spasmogenic agents. In these experiments, preparations were incubated with PIT 50 µM for 30 min and then washed in drug-free solution at the rate of 1 litre/h for 90 minutes. The tone of the preparations returned to within 20% of the original level. The dose-ratio for ATP was then found to be 6.7 ± 3.0 ($n = 6$). In another series of experiments, similar to these except that carbachol was included for the final 10 min of the exposure of the tissue to PIT, the dose-ratio for ATP 90 min after PIT was removed from the bath was 24.6 ± 9.7 ($n = 6$), this dose-ratio being significantly higher than in the former series ($P < 0.05$). The presence of spasmogens may therefore determine the degree of the antagonism of ATP by PIT, but is not a prerequisite for the antagonism to occur.

Specificity of the antagonism

In preliminary experiments, the specificity of the ATP receptor blocking action of PIT was assessed by the use of single doses of ATP, noradrenaline and isoprenaline (Hooper *et al.*, 1974). This work has been extended to include full concentration-response curves for the three agonists before and after a 30 min exposure to PIT (50 µM). The curves for noradrenaline and ATP were obtained by cumulative dosing; with isoprenaline, single doses were used and the curves constructed from the results with a large number of tissues. Carbachol (0.1-0.8 µM) was used to restore the smooth muscle tone to within 20% of the control level. The results of the experiments are shown in Figure 4. When assessed by comparison of the dose-ratios, PIT was 30 times more effective in blocking ATP-induced relaxations than noradrenaline relaxations, and 60 times more effective against ATP than against isoprenaline.

Comparison with other ATP receptor antagonists

Imidazole, phentolamine and quinidine have been used to block the inhibitory actions of ATP on isolated intestinal muscle (Bueding, Bülbring, Gercken, Hawkins & Kuriyama, 1967; Bowman & Hall, 1970; Burnstock *et al.*, 1970; Rikimaru, Fukushi & Suzuki, 1971; Satchell *et al.*, 1973; Sneddon, Smythe, Satchell & Burnstock, 1973). PIT has been compared to these other antagonists:

Table 1 The effect of spasmogens, used to restore tone, on the antagonism of adenosine 5'-triphosphate (ATP) by 2-2'pyridylisatogen (PIT)

Spasmogen	*Concentration (µM)*	*Dose-ratio (± s.e.mean)*	n
Acetylcholine	0.05- 1.0	42 ± 11	6
Carbachol	0.05- 1.0	49 ± 3	24
Histamine	0.2 - 2.0	10 ± 3	5
Prostaglandin E_2	0.3 -33.0	5 ± 2	4
None (preparations with little relaxation)		8,6,8	3

Taenia caeci preparations were incubated with PIT 50 µM for 30 min and the tone was restored to within 20% of the control level. Dose-ratios were obtained from cumulative concentration-response curves for ATP before and after exposure to the antagonist. The level of contraction was not maintained when prostaglandins were used to restore tone; thus the value in the table may be an underestimate of the potency of PIT.

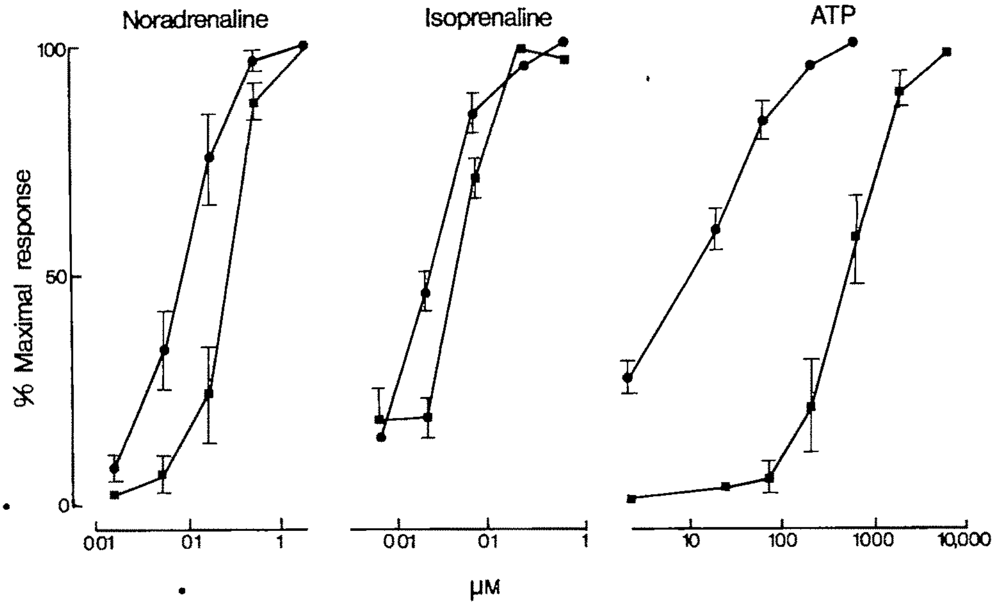


Figure 4 Specificity of the antagonism of adenosine 5'-triphosphate (ATP) by 2-2'pyridylisatogen (PIT). Concentration-response curves for ATP (8 experiments) and noradrenaline (7 experiments) were obtained by cumulative dosing and those for isoprenaline (10 experiments) by constructing concentration-response curves from single responses, one preparation being used for each response. Curves were obtained before (●) and after (■) 30 min incubation with PIT (50 µM)· vertical bars show s.e.mean. Carbachol (0.05-1.0 µM) was used to restore tone. Dose-ratios calculated from the curves were: 2.7 (noradrenaline), 1.6 (isoprenaline) and 92.6 (ATP). Relaxations induced by isoprenaline were slowed in the presence of PIT.

the results constitute Table 2. Only PIT and phentolamine significantly reduced the responses to ATP. The high concentration of phentolamine required to block the ATP-induced relaxations resulted in non-specific effects, the responses to noradrenaline, isoprenaline and ATP being similarly blocked. In the eight experiments, PIT completely blocked ATP and reduced the responses to noradrenaline (to 42% of the control) and isoprenaline (70%). Imidazole and quinidine were not antagonists of ATP at the concentrations tested.

Table 2 Specificity of drugs used to block the inhibitory effects of adenosine 5'-triphosphate (ATP)

Antagonist	*Concentration (µM)*	n	*Responses as % control (s.e.mean)* ATP	*Noradrenaline*	*Isoprenaline*
Quinidine	140	6	83(7)	1(2)	53(9)
Phentolamine	180	6	28(9)	20(12)	52(10)
Imidazole	50000	5	80(15)	43(15)	54(19)
2-2'Pyridyl-isatogen (PIT)	50	8	2(1.5)	42(13)	70(9)

Single submaximal relaxations were induced by ATP (10-40 µM), noradrenaline (0.08-2.0 µM) or isoprenaline (0.02-0.05 µM). Agonists were added to the bath, on a 5 min cycle, before and after incubation of the antagonists with the preparation for 30 minutes. Imidazole initially contracted the taenia, but the tone returned to the control level within the 30 min incubation. Carbachol (0.1-0.8 µM) was used to restore the tone with PIT and in two experiments with phentolamine.

Note that PIT was the only drug to antagonize preferentially ATP-induced relaxations.

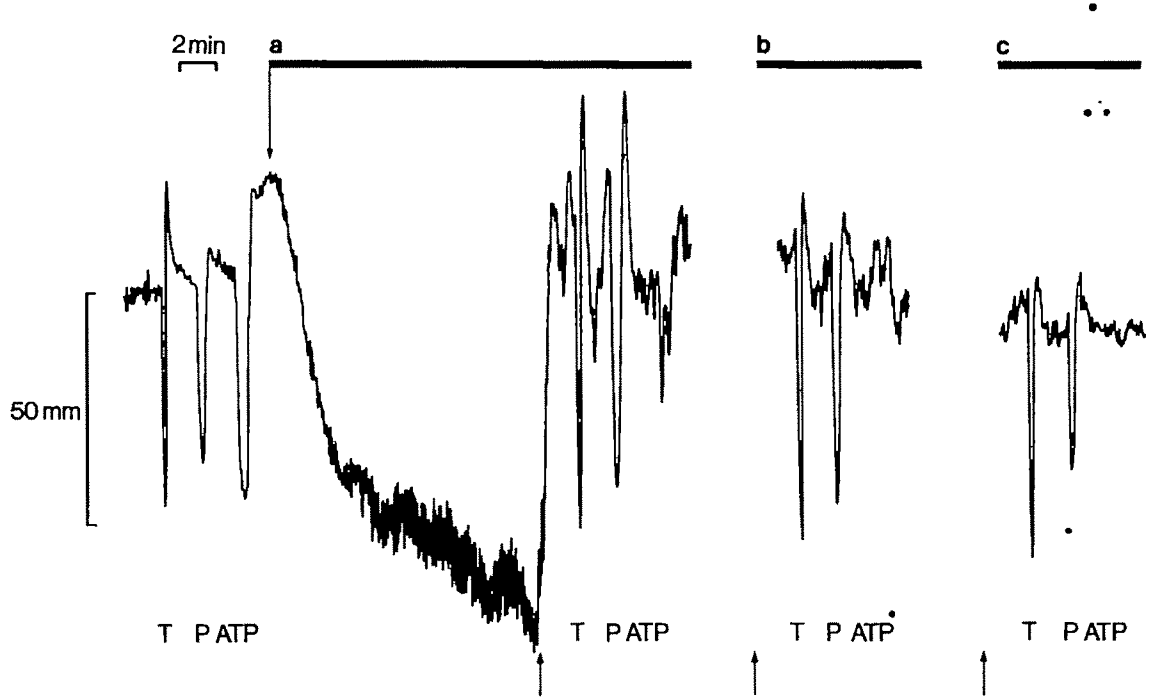


Figure 5 The effect of 50 μM 2-2'pyridylisatogen (PIT) on relaxations induced by transmural stimulation (2 Hz; 10 s; T), perivascular stimulation (50 Hz; 15 s; P) and exogenous adenosine 5'-triphosphate (20 μM; ATP). PIT was added to the bathing fluid, for the duration of the experiment, at the upper arrow; the drum speed was halved to show the resulting relaxation. Histamine was added at the lower arrows in panels (a), (b) and (c) (0.6, 0.8 and 2.5 μM respectively) and washed out between panels. There is a time interval of 30 min between panels. Hyoscine (0.33 μM) was present throughout.

The effect of PIT on transmural and perivascular nerve stimulation

The taenia caeci relaxes in response to either perivascular nerve or field stimulation in the presence of hyoscine. Figure 5 shows an experiment in which the effects of PIT were studied on both forms of stimulation and exogenous ATP. ATP was the most easily blocked, neither type of nerve stimulation being affected until the antagonist had been in contact with the tissue for at least one hour. In six experiments, after the tissue had been in contact with PIT for 30 min, the effects of perivascular nerve stimulation were 97% ± 14 of the control, transmural nerve stimulation was 114% ± 7 of the control, and ATP was 48 ± 10% (mean ± s.e.mean).

In a separate series of experiments the effects of PIT on different rates of field stimulation were compared to those on the concentration-response curves to ATP, using adjacent strips of taenia caeci. The bathing solution contained hyoscine (0.33 μM) and guanethidine (17 μM) to exclude effects on cholinoceptors or adrenergic neurones. PIT (50 μM for 30 min) blocked exogenous ATP but had little effect on the relaxations following field stimulation (Figure 6).

Discussion

In addition to blocking the effects of ATP on the guinea-pig taenia caeci, PIT relaxed the preparation. The mechanism of this relaxation is unknown, but preliminary experiments indicate that it is unlikely to be due to an action on nerves, adrenoceptors, ATP receptors or by inhibition of prostaglandin biosynthesis. These results and those of experiments with analogues of PIT (Hooper & Spedding: unpublished observations) show that independent mechanisms are involved in these two actions of PIT.

The relaxation of the taenia caeci by PIT necessitated the use of spasmogenic agents in most experiments. Although the degree of the ATP receptor blockade was influenced by the individual spasmogen, the presence of a smooth muscle

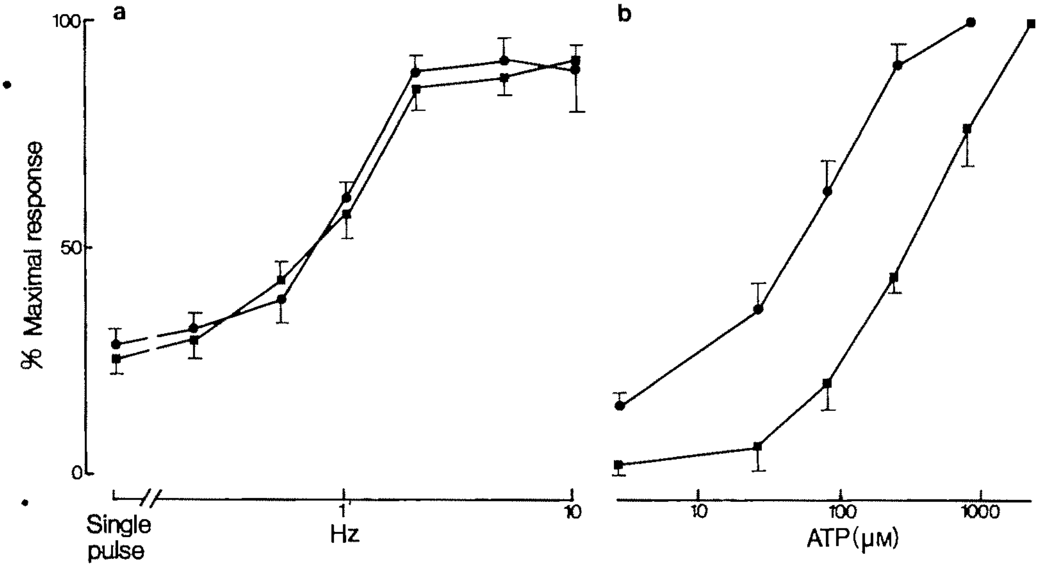


Figure 6 The effects of (a) transmural stimulation of adjacent strips of guinea-pig taenia caeci and (b) of exogenous adenosine 5′-triphosphate (ATP). The McEwen's solution contained hyoscine (0.33 μM) and guanethidine (17 μM). Responses to ATP were obtained cumulatively; adjacent strips were stimulated transmurally on an ascending series from 0.2-10 Hz every 2 minutes. Responses before (●) and after (■) incubation of the preparations with 2-2′pyridylisatogen (50 μM) for 30 minutes. Vertical bars show s.e.mean (*n* = 6). Histamine was used to restore tone. Note that although responses to ATP were diminished, the responses to transmural stimulation were unchanged.

stimulant was not a prerequisite for the antagonism to occur.

The antagonism of ATP by PIT was reversible in the early stages, so it is possible that PIT initially combines with ATP receptors in a competitive manner. However, after a period of time that depended upon the concentration of PIT, the antagonism became irreversible. Thus, although it may seem feasible, the data obtained from concentration-response curve experiments should not be used to calculate a pA_2 value for PIT because these constants depend upon measurements made during a reversible antagonism with equilibria established between the antagonist and the receptors and the agonist and the receptors. Nevertheless, using the Arunlakshana & Schild (1959) method, PIT has an apparent pA_2 of 4.8, but the slope of the plot ($\log_{10}$ agonist dose-ratio-1 against $\log_{10}$ reciprocal of the molar concentration of the agonist) was 2.6, and not the hypothetical value for a competitive antagonist of 1.0. Thus, under these conditions, PIT was not a competitive antagonist of ATP. Hooper & Robertson (1971) have shown that PIT is capable of forming covalent bonds with certain amino acids, so it is possible that PIT could be reacting with such an amino acid located at, or near, the ATP receptors in the taenia caeci to produce the irreversible blockade.

PIT was clearly a better antagonist of the inhibitory effects of ATP than were those drugs used in previous studies. Quinidine and imidazole did not significantly block the effects of ATP. Phentolamine, although it did block the effects of ATP, also blocked the inhibitory effects of noradrenaline and isoprenaline. PIT was the only drug to antagonize differentially the ATP-induced relaxations of the smooth muscle. In this series of experiments, quinidine was found to possess appreciable α-adrenoceptor antagonist activity, as had been reported previously (Hiatt, 1950; Bowman & Hall, 1970; Schmid, Nelson, Mark, Heistad & Abboud, 1974).

Relaxation of the taenia caeci induced by field stimulation in the presence of hyoscine and guanethidine, a procedure which should specifically excite the hypothetical purinergic nerves, was not reduced by PIT. Even after an extended incubation of the taenia caeci with several concentrations of PIT and with a range of stimulation frequencies, there was no reduction in the effects of field stimulation. If ATP is the mediator of the hyoscine- and guanethidine-resistant inhibitory effects of field stimulation, why was the response to field stimulation unaffected? One explanation is that ATP may be released into the synaptic cleft in such high local concentrations that PIT is ineffective. This is improbable when one considers that PIT produces an irreversible, non-competitive inhibition. However, Tomita & Watanabe (1973), using the taenia caeci in a double sucrose gap assembly, have reported that injections of ATP 1 mM matched the hyperpolarization produced by field stimulation with a single pulse. If such high concentrations of ATP occur in the synaptic cleft under physiological conditions one would expect to find 'close-contact' varicosities on electronmicrographs of the tissue so that the ATP is used efficiently. These have rarely been observed in the taenia caeci (Bennett & Rogers, 1967). Also, the long latency of the inhibitory junction potential (Bennett, Burnstock & Holman, 1966) suggests that the neurotransmitter diffuses over a considerable distance (100-300 nm) before reaching the receptors on the smooth muscle cells. Thus it would appear unlikely that ATP would reach such high concentrations in the synaptic cleft. However, it must be acknowledged that the highest concentration of the transmitter would occur in the centre of the muscle bundle where, presumably, the concentration of PIT is lowest.

The results in this paper are at variance with the purinergic nerve hypothesis. However, the acceptance or rejection of the hypothesis must be postponed until there is more evidence about the release of purine nucleotides from the taenia caeci and other tissues.

We are grateful to our colleague Dr M. Hooper, for synthesizing the 2-2'pyridylisatogen tosylate; to Dr R.T. Brittain, Allen and Hanburys Ltd., Ware, Herts., for stimulating discussions throughout the course of this work; and to Mr D.W. Snowdon for photographing the figures. We would also like to thank Dr J.E. Pike, The Upjohn Co., Kalamazoo, Michigan, for a gift of prostaglandin E_2.

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COMBINED α- AND β-ADRENOCEPTOR BLOCKING DRUG AH 5158: FURTHER STUDIES ON α-ADRENOCEPTOR BLOCKADE IN ANAESTHETIZED ANIMALS

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1 AH 5158, 5-[1-hydroxy-2-[(1-methyl-3-phenylpropyl)amino]ethyl]salicylamide, competitively antagonised phenylephrine-induced vasopressor responses in anaesthetized dogs, thus confirming that the drug possesses α-adrenoceptor blocking activity.

2 In contrast, AH 5158 was a relatively ineffective antagonist of vasopressor responses to noradrenaline in anaesthetized dogs. Thus, at the lowest dose-level tested (1 mg/kg) AH 5158 abolished the increase in pulse width caused by noradrenaline, but otherwise had little or no blocking effect in doses as high as 10 mg/kg. Propranolol (0.1 mg/kg) also abolished the increase in pulse width caused by noradrenaline. With both drugs this effect is thought to be a consequence of blockade of the β-adrenoceptor-mediated cardiac stimulant action of noradrenaline.

3 The interaction between AH 5158 and noradrenaline in spinal dogs, anaesthetized cats and pithed rats was very similar to that seen in anaesthetized dogs.

4 Noradrenaline pressor responses *were* effectively antagonized by AH 5158 in anaesthetized dogs pretreated with cocaine. The degree of block was similar to that obtained when phenylephrine was the agonist in untreated dogs.

5 These results are consistent with the hypothesis that AH 5158 blocks a cocaine-sensitive inactivation process for noradrenaline in addition to blocking α- and β-adrenoceptors. The resultant increase in the level of circulating noradrenaline would tend to counteract the adrenoceptor blocking action of the drug.

6 The implications of these findings are discussed.

Introduction

AH 5158, 5-[1-hydroxy-2-[(1-methyl-3-phenylpropyl)amino]ethyl] salicylamide, possesses a unique combination of α- and β-adrenoceptor blocking activity (Farmer, Kennedy, Levy & Marshall, 1972). The α-adrenoceptor blocking action of the drug in the anaesthetized dog was unusual in that vasopressor responses to noradrenaline were blocked to a much lesser extent than those to oxymetazoline. This difference was abolished by pretreating dogs with cocaine. To explain these results it was suggested that AH 5158 blocks a cocaine-sensitive inactivation process for noradrenaline, enabling a larger proportion of the administered dose of noradrenaline to compete with AH 5158 at α-adrenoceptors and thereby reducing the degree of observed antagonism (Farmer *et al.*, 1972).

This paper describes the results of further studies on the interaction between AH 5158 and α-adrenoceptor agonists on the blood pressure and heart rate of anaesthetized animals. These interactions have been re-examined in greater detail in the anaesthetized dog and the investigations have been extended to the spinal dog, anaesthetized cat and the pithed rat.

Methods

Preparation of animals

Beagle dogs of either sex weighing 7-14 kg were anaesthetized with thiopentone sodium, 25 mg/kg intravenously, followed by barbitone sodium, 250 mg/kg intraperitoneally. In some experiments, dogs were anaesthetized with 3% halothane in a nitrous oxide : oxygen (3 : 1) mixture and made spinal by the method of Burn (1952). Artificial respiration with room air was applied through a cuffed endotracheal tube using a stroke volume of

13 ml/kg and a rate of 28/minute. In these experiments and those described below, arterial blood pressure (1 mmHg ≈ 133 Pa) was measured from a cannula in a femoral or carotid artery with a Bell and Howell transducer (type 4-327-L221) and heart rate was obtained from a cardiotachometer triggered by the arterial pressure signal.

Cats of either sex weighing 2.0-3.5 kg were anaesthetized with chloralose, 80 mg/kg intravenously, after induction with 3% halothane in a nitrous oxide : oxygen (3 : 1) mixture. Animals were respired artificially with room air through a tracheal cannula using a stroke volume of 13 ml/kg and a rate of 28/minute.

Male albino rats were anaesthetized with 3% halothane in a nitrous oxide : oxygen (3 : 1) mixture and pithed as described by Shipley & Tilden (1947). The animals were respired artificially with room air through a tracheal cannula using a stroke volume of 1 ml/100 g and a rate of 50/minute.

In all experiments drugs were administered through a cannula in a femoral vein. Bilateral vagotomy was performed in all anaesthetized animals. Permanent records of experiments were made on a Devices M8 recorder.

Experimental procedure and analysis of results

Unless stated otherwise, the following procedure was used to examine agonist-antagonist interactions in each animal. Vasopressor dose-response curves were obtained by the sequential intravenous injection of increasing doses of either noradrenaline or phenylephrine. Each dose-response curve was made up of four or five doses of agonist administered at intervals of 10-20 min and took about 45 min to complete. Dose-response curves were repeated at intervals of approximately 1 hour. After two control dose-response curves had been obtained, the first dose of AH 5158 was injected and the vasopressor dose-response curve re-determined, beginning 15 min later. Three dose-levels of AH 5158 were examined in each experiment, the doses being given at intervals of approximately 1 hour. A cumulative dosing schedule was used for AH 5158; for example, to increase the dose-level from 3 mg/kg to 10 mg/kg a further dose of 7 mg/kg was administered. Dose-ratios were estimated by comparing the dose-response curves obtained in the presence of AH 5158 with the control dose-response curves. The level of response was selected in each experiment to ensure that comparison was made on the straight-line part of a dose-response curve. When appropriate, the results were expressed graphically by plotting log (agonist dose-ratio −1) on log antagonist dose (mg/kg i.v.) (Arunlakshana & Schild, 1959).

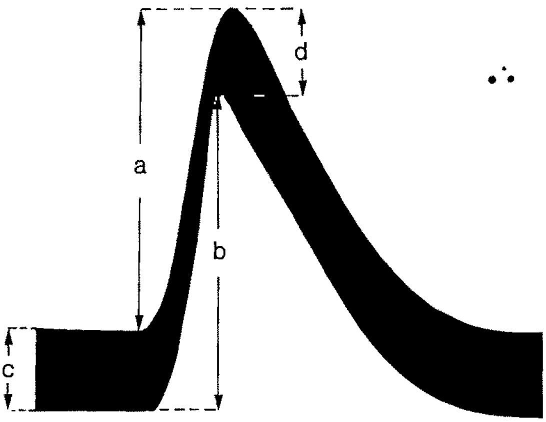

Figure 1 Diagramatic representation of a vasopressor response. The parameters used for analysis were (a) systolic pressor response (b) diastolic pressor response (c) pulse width prior to vasopressor response (d) pulse width at peak of pressor response.

Assessment of the results also involved a detailed analysis of the pressor responses to α-adrenoceptor agonists. The various parameters measured are illustrated diagrammatically in Figure 1.

Drugs

The following drugs were used: AH 5158 hydrochloride (Allen & Hanburys); cocaine hydrochloride (MacFarland Smith); (−)-noradrenaline (BDH); (−)-phenylephrine hydrochloride (Koch-Light); (±)-propranolol hydrochloride (ICI). Solutions were prepared by dissolving drugs in 0.9% w/v NaCl solution. Weights of drugs are expressed in terms of the base.

Results

Anaesthetized dogs

Variation in sensitivity to noradrenaline and phenylephrine In control experiments vasopressor dose-response curves to noradrenaline 0.1-3 μg/kg ($n = 3$) or phenylephrine 1-30 μg/kg ($n = 3$) were obtained at intervals of 1 h over a period of 4-6 hours. Sensitivity to noradrenaline increased by a maximum of 2- to 3-fold over this period; this change was considered to be negligible. There was no consistent change in sensitivity to phenylephrine.

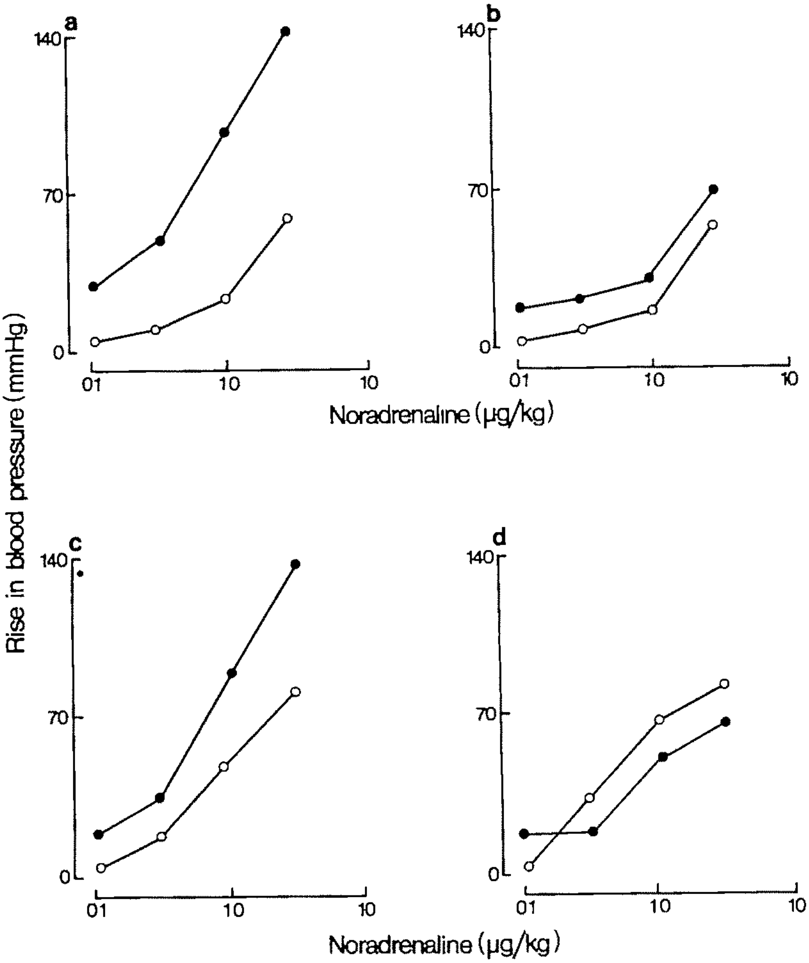

Figure 2 Anaesthetized dog. Pressor responses to noradrenaline; (a) and (c) are control responses; (b) effects of AH 5158 (1 mg/kg) and (d) of propranolol (0.1 mg/kg) on responses to noradrenaline. (ab) and (cd) are the results of single experiments Systolic pressor response (●), diastolic pressor response (○). All drugs given intravenously. Note that both antagonists reduce systolic pressor responses but have little effect on diastolic pressor responses.

Interaction between noradrenaline and AH 5158 or propranolol The systolic pressor response to noradrenaline was always larger than the diastolic pressor response and this difference increased with increasing dose. Thus, noradrenaline caused dose-dependent increases in pulse width.

In four dogs AH 5158, 1 mg/kg, reduced systolic pressor responses to noradrenaline, but had little effect on diastolic pressor responses. After AH 5158, the systolic and diastolic pressor responses were similar in magnitude so that the increase in pulse width was abolished. The results of one experiment are summarized in Figure 2a, b.

In three experiments propranolol, 0.1 mg/kg, had very similar effects to AH 5158, reducing systolic but not diastolic pressor responses to noradrenaline (Figure 2c,d). Higher doses of AH 5158 (3 and 10 mg/kg) modified systolic and diastolic pressor responses in the same way and so the results obtained with the latter only are described. In two experiments these higher doses of AH 5158 slightly antagonized diastolic pressor responses (maximum noradrenaline dose-ratio, 4-5), but the antagonism was not dose-related. In the other two experiments AH 5158 had no effect on diastolic pressor responses. The results of one experiment

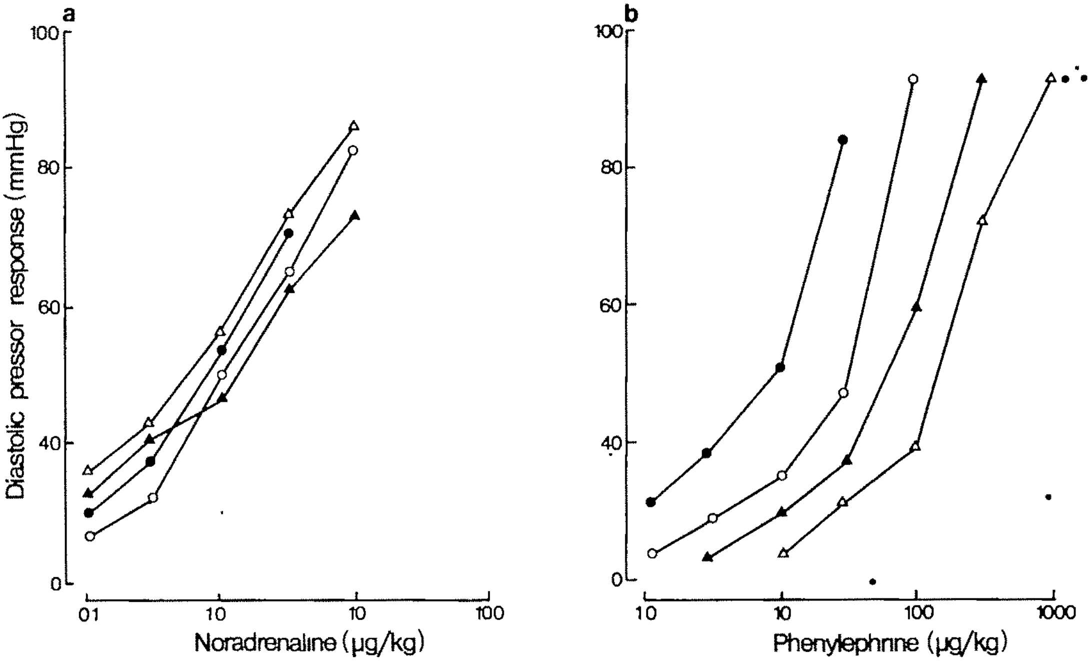

Figure 3 Anaesthetized dog Comparison of the effect of AH 5158 on diastolic pressor responses to noradrenaline (a) and phenylephrine (b). (a) and (b) are results from individual experiments. Control (●) and after AH 5158, 1 mg/kg (○), 3 mg/kg (▲), 10 mg/kg (△). All drugs given intravenously.

illustrating the interaction between noradrenaline diastolic pressor responses and AH 5158, 1, 3 and 10 mg/kg, are shown in Figure 3a.

The pressor responses to noradrenaline were accompanied by decreases in heart rate of up to 20 beats/min, but these were not dose-related. In some dogs small increases in heart rate preceded the decreases. Both were abolished by AH 5158, 1 mg/kg, or by propranolol, 0.1 mg/kg.

Interaction between phenylephrine and AH 5158 The vasopressor responses to phenylephrine differed from those to noradrenaline in that systolic and diastolic pressor responses were similar in size. Consequently, pulse width was not increased by phenylephrine.

The effect of AH 5158, 1, 3 and 10 mg/kg, on pressor responses to phenylephrine was examined in three dogs. Systolic and diastolic pressor responses were blocked to a similar extent and so only results for the latter are described. AH 5158 caused dose-related parallel shifts to the right of the phenylephrine dose-response curve. In Figure 3b the results of a typical experiment are contrasted with those obtained with noradrenaline (Figure 3a). The combined results of the three experiments with phenylephrine are expressed in the form of an Arunlakshana & Schild (1959) plot in Figure 4a.

Pressor responses to phenylephrine were accompanied by decreases in heart rate of 10-30 beats/minute. These decreases were abolished by AH 5158, 1 mg/kg.

Interaction between noradrenaline and AH 5158 in cocaine-pretreated dogs Preliminary experiments were directed towards determining the dose of cocaine required for maximum potentiation of noradrenaline pressor responses. The degree of potentiation increased with doses of cocaine up to 10 mg/kg. At 30 mg/kg, cocaine caused pronounced cardiac arrhythmias and hypotension, resulting in death. Therefore, cocaine was used at the 10 mg/kg dose-level in these experiments. This dose, infused over a period of 15-20 min, increased diastolic blood pressure by 15-40 mmHg and heart rate by 10-40 beats/minute. On termination of the infusion, blood pressure and heart rate stabilized at levels slightly below control within 10-15 minutes. The potentiating action of cocaine began to wane after 2 h and so the effect of only one dose of AH 5158 was examined in each dog. The

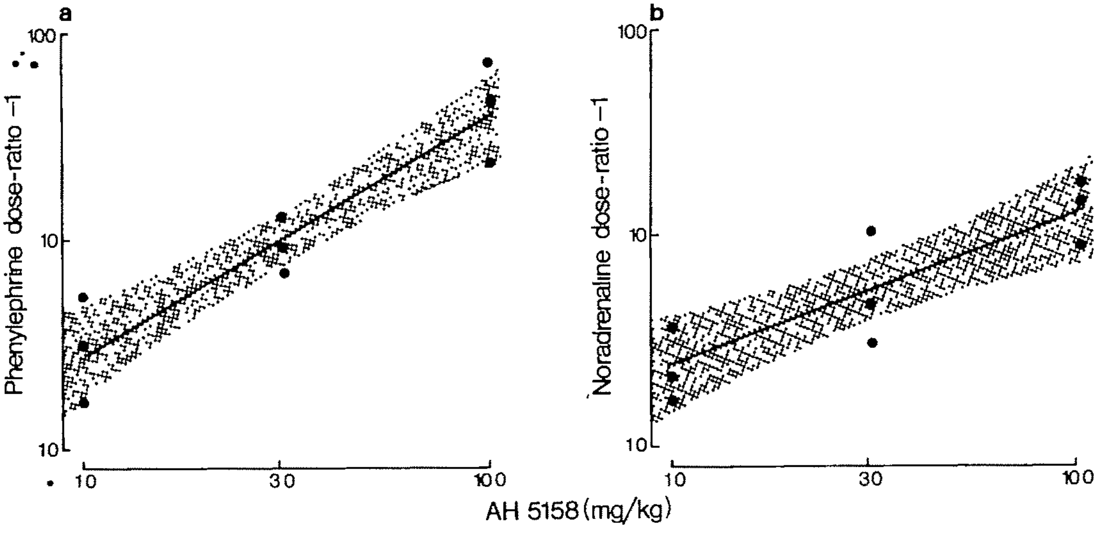

Figure 4 Arunlakshana & Schild (1959) plots computed for the interaction between (a) AH 5158 and phenylephrine in anaesthetized dogs and (b) AH 5158 and noradrenaline in anaesthetized dogs pretreated with cocaine, 10 mg/kg i.v. Each point represents a single estimate of dose-ratio −1. In (a) AH 5158 was administered at three dose-levels in each of three dogs. In (b) only one dose of AH 5158 was administered to each dog. The shaded envelopes enclose the 95% confidence limits of the regression lines. The limits for the two regression lines overlap. All drugs given intravenously

following procedure was used: a pressor dose-response curve was obtained for noradrenaline, cocaine was infused over a period of 15-20 min to give a total dose of 10 mg/kg, a further 20 min was allowed to elapse and a second noradrenaline dose-response curve was obtained; AH 5158, 1, 3 or 10 mg/kg, was injected and 15 min later a third noradrenaline dose-response curve was obtained. The potentiating effect of cocaine was estimated by comparing the first and second noradrenaline dose-response curves; the blocking effect of AH 5158 was estimated by comparing the second and third noradrenaline dose-response curves. Diastolic pressor responses were used to construct dose-response curves. Each dose-level of AH 5158 was tested in three dogs.

Sensitivity to noradrenaline was greatly increased by cocaine, the dose-response curve being displaced to the left by a factor of 15 (95% confidence limits 8-27, $n = 9$). In these animals the subsequent administration of AH 5158, 1, 3 or 10 mg/kg, produced parallel dose-related displacements to the right of the noradrenaline dose-response curve. This contrasts with the results obtained in the absence of cocaine (see above). Results for these experiments are summarized in the form of an Arunlakshana & Schild (1959) plot in Figure 4b. Comparison of Figure 4a and b shows that the degree of α-adrenoceptor blockade produced by AH 5158 is similar when phenylephrine is the agonist in untreated dogs and when noradrenaline is the agonist in cocaine-treated dogs.

In two experiments cocaine, 10 mg/kg, had no effect on sensitivity to phenylephrine pressor responses.

Spinal dogs

In four spinal dogs pressor responses to noradrenaline, 0.1-3 μg/kg, were very similar in character to those obtained in anaesthetized dogs. Systolic pressor responses were larger than diastolic pressor responses and, consequently, pulse width was increased. The effect of AH 5158 on noradrenaline pressor responses was also very similar to that seen in anaesthetized dogs. Thus, at 1 mg/kg AH 5158 reduced systolic but not diastolic pressor responses and abolished the increases in pulse width. Larger doses of AH 5158 (3 and 10 mg/kg) had no further blocking effect in three dogs. In the fourth dog the diastolic pressor dose-response curve was displaced to the right by a maximum of 4-fold, but this antagonism was not dose-related.

Pressor responses to noradrenaline were accompanied by dose-related increases in heart rate of up

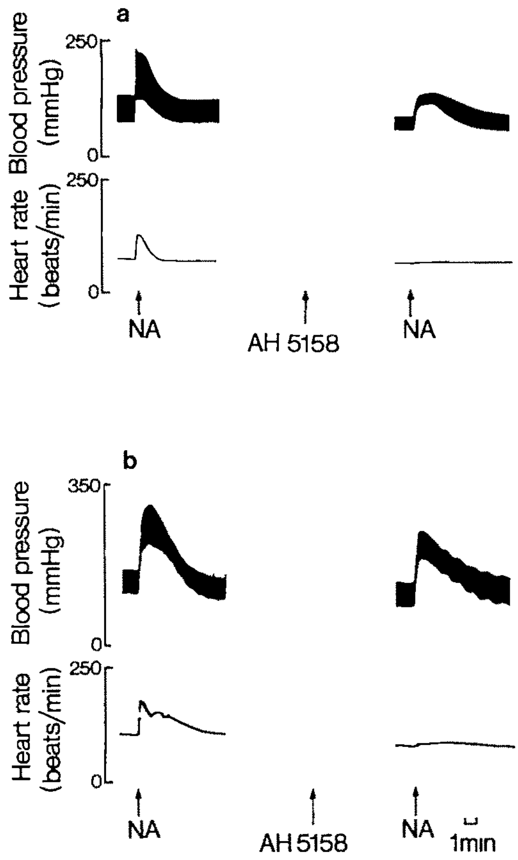

Figure 5 Comparison of the effects of AH 5158, 3 mg/kg, on responses of blood pressure and heart rate to noradrenaline, 3 μg/kg (NA), in (a) the spinal dog and (b) the anaesthetized cat. Drugs given intravenously.

to 100 beats/min, in contrast to the decreases or biphasic responses seen in the anaesthetized dog. These heart rate increases were abolished by AH 5158, 1 mg/kg.

The effects of AH 5158, 1 mg/kg, on the blood pressure and heart rate responses to noradrenaline, 3 μg/kg, are illustrated in Figure 5a.

Anaesthetized cats

In three anaesthetized cats the responses to noradrenaline, 0.1-3 μg/kg and the interaction between noradrenaline and AH 5158, 1-10 mg/kg, on blood pressure and heart rate were almost identical to those obtained in anaesthetized and spinal dogs. Records from one experiment in an anaesthetized cat are shown in Figure 5b.

Pithed rats

In three pithed rats the responses to noradrenaline, 0.1-3 μg/kg, and the interaction between noradrenaline and AH 5158, 1-10 mg/kg, on blood pressure and heart rate were almost identical to those obtained in anaesthetized and spinal dogs and anaesthetized cats.

Discussion

In a previous study in the anaesthetized dog, AH 5158 exhibited α-adrenoceptor blocking activity when oxymetazoline was the agonist used (Farmer *et al.*, 1972). This activity has been confirmed in the present study using phenylephrine. Comparison of the two series of experiments shows that vasopressor responses to these agonists were antagonized to a similar degree by AH 5158.

In contrast, AH 5158 was a relatively ineffective antagonist of vasopressor responses to noradrenaline, which confirms the previous finding (Farmer *et al.*, 1972). The more detailed analysis reported here gives rise to two questions. First, why does AH 5158, at the lowest dose-level tested (1 mg/kg), abolish the increases in pulse width caused by noradrenaline, and second, why does AH 5158 otherwise have little or no blocking effect in doses as high as 10 mg/kg?

The answer to the first question probably lies in the dual origin of the noradrenaline pressor response. This consists of a peripheral vasoconstrictor component mediated *via* α-adrenoceptors and a cardiac component (positive chronotropic and/or positive inotropic) mediated *via* β-adrenoceptors. The cardiac component appears to be responsible for the increase in pulse width; thus, phenylephrine, which has much less cardiac stimulant action than noradrenaline (Trendelenburg, Muskus, Flemming & Gomez Alonso de la Sierra, 1962; Shanks, 1966) caused no increase in pulse width. AH 5158 exerts a pronounced block of cardiac β-adrenoceptors at 1 mg/kg (Farmer *et al.*, 1972; this study) and this action is probably responsible for preventing the increases in pulse width to noradrenaline. The almost identical results obtained for the interaction between noradrenaline and propranolol provide strong support for this idea.

The second question can be answered by referring to the experiments carried out in dogs treated with cocaine. In these animals noradrenaline pressor responses *were* effectively antagonized by AH 5158, thus confirming our previous finding (Farmer *et al.*, 1972). In the experiments reported here, care was taken to minimize the influence of cocaine-sensitive uptake mechanisms. The block of noradrenaline pressor responses by AH 5158 was then similar in degree to the block of phenylephrine responses in untreated dogs. These findings are consistent with the hypothesis that the α-adrenoceptor blocking action of AH 5158 is normally counteracted when noradrenaline is the agonist, because AH 5158 also blocks a cocaine-sensitive inactivation mechanism for noradrenaline. At each dose-level the increase in the level of circulating noradrenaline is apparently just sufficient to counteract the α-adrenoceptor blockade. This situation does not arise when phenylephrine is the agonist because it is not inactivated to any significant degree by these mechanisms, as indicated by the lack of potentiation of pressor responses by cocaine. We intend to test the hypothesis of a 'cocaine-like' action of AH 5158 more directly by comparing the blood levels of intravenously administered noradrenaline before and after administration of AH 5158.

Blockade of noradrenaline inactivation mechanisms by AH 5158 would also be expected to influence its β-adrenoceptor blocking action when noradrenaline is the agonist. However, because AH 5158 is some 4 to 5 times more potent as an antagonist at β-adrenoceptors than at α-adrenoceptors (Farmer *et al.*, 1972) the increases in the levels of circulating noradrenaline are probably not sufficient to overcome the β-adrenoceptor blockade. The present study bears this out since AH 5158 effectively antagonized the cardiac stimulant actions of noradrenaline.

The present experiments do not indicate the location of the cocaine-sensitive inactivation process thought to be blocked by AH 5158. There are two likely sites; first, the peripheral vascular beds, where noradrenaline is removed from the circulation into sympathetic nerve terminals by the uptake$_1$ process (Whitby, Hertting & Axelrod, 1960; Whitby, Axelrod & Weil-Malherbe, 1961; Gryglewski & Vane, 1970; Iversen, 1971), and second, the lungs, where noradrenaline is removed from the circulation by an uptake process sensitive to cocaine, but which otherwise differs from uptake$_1$ (Alabaster & Bakhle, 1973; Iwasawa & Gillis, 1974).

The lack of antagonism of noradrenaline pressor responses by AH 5158 is not restricted to the anaesthetized dog; it was also demonstrated in the spinal dog, anaesthetized cat and pithed rat. It may also explain differences in the pharmacological results obtained with AH 5158 in man. Thus, in human volunteers Boakes, Knight & Prichard (1971) found AH 5158 to be about 4 times less potent as an antagonist at α-adrenoceptors than at β-adrenoceptors, whereas Collier, Dawnay, Nachev & Robinson (1972) found it to be 40 to 200 times less potent. It may be relevant that the former group used phenylephrine as the α-adrenoceptor agonist whereas the latter group used noradrenaline.

The ineffectiveness of AH 5158 against systemically administered noradrenaline raises a doubt about its ability to block responses to noradrenaline released from sympathetic nerves. This question has obvious clinical implications. We have shown that AH 5158 *does* effectively block responses to sympathetic nerve stimulation in the heart and peripheral vasculature of the dog (unpublished observations). It has also been shown to antagonize responses to cardiac sympathetic nerve stimulation in man (Collier *et al.*, 1972). This difference in the ability of AH 5158 to antagonize systemically administered and neuronally released noradrenaline remains to be explained.

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EFFECT OF FLUPHENAZINE ON TISSUE NORADRENALINE CONCENTRATIONS AND ITS INTERACTION WITH PARGYLINE

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Fluphenazine caused a small decrease in the noradrenaline (NA) concentrations of the rat brain, possibly by impairing granular amine storage. The drug diminished the rise in brain and heart NA concentrations induced by pargyline, suggesting that it might possess inhibitory properties on neuronal uptake mechanisms and/or NA synthesis. Fluphenazine abolished conditioned avoidance responses in rats, an effect which was maintained after the concomitant administration of pargyline, when NA concentrations remained high. This suggests that the fluphenazine-induced sedation is not mediated via its effect on brain NA content, but is possibly due to the effect of the drug on NA turnover rates in the brain.

Introduction Fluphenazine is a very potent phenothiazine antipsychotic agent (Hanlon, Michaux, Ota, Shaffer & Kurland, 1965), capable of abolishing conditioned avoidance responses (CAR) and of taming animals (High, Hassert, Rubins, Piala, Burke & Graver, 1960). Since behavioural depressions are often associated with an absolute, or relative deficiency of brain catecholamines, particularly noradrenaline (NA) (Schildkraut, 1965), it was of interest to investigate the effect of the drug on the NA concentration in the central nervous system. For the measurement of the behavioural depressions, use was made of the fact that neuroleptic drugs disrupt conditioned responses (avoidance) without necessarily blocking the unconditioned ones (escape) (Cook & Weidley, 1957). We also studied whether the behavioural depression caused by fluphenazine could, like that induced by reserpine, be effectively reversed by the monoamine oxidase (MAO) inhibitor, pargyline. The NA concentration in the hearts and brains of animals treated with a combination of both drugs was also measured.

Methods

Tissue noradrenaline concentrations Male Wistar rats (120-150 g) were divided into three groups of 24 rats each. One group received a single dose of fluphenazine dihydrochloride (Squibb), 2 mg/kg, the second group pargyline hydrochloride (Abbott) 100 mg/kg and the third group pargyline and fluphenazine simultaneously in the doses stated above. The drugs were injected subcutaneously. A further group of rats was given 0.9% w/v NaCl solution (saline) and served as controls. From each group eight rats were killed at 1 h, eight at 3 h and eight at 24 h after the treatment. The whole brain and heart were isolated. For each sample to be analysed, the hearts or brains of two rats were pooled and their total catecholamine content was estimated as 'NA' by the method of Anton & Sayre (1962).

Conditioned avoidance responses The apparatus used consisted of a four-compartment cage, with a safety area at one end and an electrifiable grid floor in the other compartments. The compartments were separated by glass panels such that the rat had to follow a zigzag path from one end of the cage (electrified) to the other (safety area). Each animal was left in the cage for 15 min to acclimatize it before conditioning. It was then placed at one end of the cage and the electric stimulator turned on. This consisted of an automatic unit, sounding a buzzer for 4 s and then delivering an electric current (60 V at a frequency of 25 Hz) for 20 seconds.

After several trials, the rat learned to respond to the sound stimulus alone, by crossing the cage to the safety area. Such animals were considered as 'conditioned', whereas those which failed to respond in this way were rejected. Ten trials were performed before drug administration allowing a 5 min rest period between the trials. Only those animals which succeeded in responding to the conditioned stimulus at least 9 times out of 10 trials were used for testing. The conditioned rats were divided into groups of 10 and their CAR was tested 1, 3 and 24 h after drug administration. The percentage of animals failing to respond to the conditioned stimulus was recorded. The drugs were injected subcutaneously in doses of 2 mg/kg for fluphenazine and 100 mg/kg for pargyline.

Results

Effect of drugs on noradrenaline concentrations As shown in Table 1, fluphenazine caused a significant reduction (44%) in brain NA concentra-

Table 1 The effect of fluphenazine (2 mg/kg, s.c.) and pargyline (100 mg/kg, s.c.) given singly or in combination, on the noradrenaline content of rat brain and heart

	Brain (μg/g wet tissue)			*Heart (μg/g wet tissue)*		
	1 h	*3 h*	*24 h*	*1 h*	*3 h*	*24 h*
Fluphenazine	0.45	0.234	0.35	1.39	1.48	1.13
	±0.03	±0.02	±0.033	±0.08	±0.18	±0.043
Control	0.51	0.42	0.45	1.24	1.24	1.06
	±0.008	±0.008	±0.02	±0.076	±0.076	±0.04
% change	−13.33	−44.28**	−22.20*	+12.09	+19.35	+7.62
Pargyline	0.43	0.89	1.16	1.75	2.02	3.70
	±0.045	±0.003	±0.024	±0.10	±0.05	± 0.38
Control	0.46	0.41	0.41	1.59	1.85	1.75
	±0 03	±0.04	±0.041	±0.07	±0.15	±0.19
% change	−6.52	+117.07**	+182.93**	+10.06	+9.19	+111.43**
Fluphenazine plus pargyline	0.59	0.56	0.84	1.96	1.92	2.81
	±0.07	±0.006	±0.06	±0.06	±0.04	±0.26
Control	0.44	0.37	0.44	1.92	1.92	1.59
	±0.02	±0.006	±0.04	±0.08	±0.11	±0.07
% change	+33.65	+53.15**	+90.90**	+2.08	–	+76.73**

Each value represents the mean for 8 animals ± s.e.mean.
* $P < 0.05$ compared with control; ** $P < 0.01$ compared with control.

tion, 3 h after its administration. The value tended to return to normal after 24 hours. The drug failed to show any effect on the heart NA content during the same experimental period.

Pargyline caused a progressive rise in the brain and heart NA concentration. After 24 h the values were nearly trebled or doubled respectively. The simultaneous injection of both drugs resulted in an increase in the brain NA concentrations by 50% after 2 h and 90% after 24 hours. An increase of 77% in the heart NA content was observed only after 24 hours.

Effect of drugs on conditioned avoidance responses CAR was abolished in all animals 1 h after administration of fluphenazine. This effect was maintained for 3 hours. After 24 h only 20% of the animals still showed a loss of CAR. There was no observable impairment of motor activity in the fluphenazine-treated animals. They all responded to the unconditioned stimulus. The result of the combined treatment with fluphenazine and pargyline was essentially the same as that with fluphenazine alone. All animals lost the CAR 1 h after drug administration, and the effect was still present 2 h later. After 24 h recovery occurred only in 10% of the animals which had shown a loss of the CAR.

Discussion The reduction in the brain NA concentrations caused by fluphenazine is probably due to an impairment of the storage of NA within the granules, as was previously reported for other phenothiazine derivatives (Gey & Pletscher, 1964). This may, or may not be accompanied by an inhibition of the neuronal uptake of NA (Schanberg, Schildkraut & Kopin, 1967). These two actions of phenothiazine derivatives may be mediated via their inhibitory actions on flavoenzymes (Gabay & Harris, 1966; 1967) possibly by decreasing neuronal adenosine triphosphate (ATP) content. In the heart, no change in NA content was observed, possibly as a result of a balance between the factors influencing synthesis and release. A rise in the NA content of brain and heart after pargyline has been reported by other authors (Schoepke & Wiegand, 1963; Spector, Hirsch & Brodie, 1963). This rise may be due to an increase in the endogenous NA content as a result of inhibition of intraneuronal MAO. When fluphenazine was combined with pargyline, fluphenazine diminished the rise in NA caused by pargyline alone, in both the brain and the heart. Such an interaction has been previously reported by Gey & Pletscher (1961) in the brain using chlorpromazine and other MAO inhibitors. Assuming that fluphenazine possesses an inhibitory effect on neuronal uptake of NA, as has been attributed to other phenothiazines (Schanberg *et al.*, 1967), then the increase of the endogenous NA concentration induced by pargyline could be suppressed by it.

From the behavioural experiments, it could be seen that the onset of the tranquillizing action of fluphenazine preceded the onset of the NA reduction in the brain. On combining fluphenazine with pargyline, the results show that pargyline offered no protection against the fluphenazine-induced disruption of CAR. Moore & Rech (1967) using a rotarod conditioning method have reported similar findings with fluphenazine and the MAO inhibitor, tranylcypromine. Since the net effect of such a combination was a rise in brain NA concentration, it would seem that the fluphenazine-induced sedation is not entirely dependent on the NA content of the brain. It might depend on other brain neurohumoral agents such as dopamine or 5-hydroxytryptamine. However, this seems unlikely since the breakdown of these amines is also dependent on MAO activity (Pletscher, 1966). More probably the effect is linked with changes in the turnover rates of NA (Neff & Costa, 1968). Further work is being carried out to elucidate our present findings.

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INDOMETHACIN POTENTIATION OF THE RESPONSE OF THE RABBIT PORTAL VEIN TO ELECTRICAL STIMULATION: EFFECT OF STIMULUS FREQUENCY AND TRAIN LENGTH

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The response of the rabbit isolated portal vein to electrical stimulation was potentiated by indomethacin (10 μg/ml). This potentiation was dependent upon frequency but independent of the number of pulses in a train. It is concluded that these results add further support for the idea that indomethacin increases the release of newly synthesized noradrenaline as a result of inhibition of prostaglandin synthesis.

Introduction The contractile response of the rabbit portal vein to electrical stimulation is inhibited by prostaglandin E_2 and potentiated by indomethacin. Both the inhibition and potentiation are frequency-dependent. It was postulated that the noradrenaline which is released in response to low frequencies of stimulation is newly synthesized, and that prostaglandins control this release (Greenberg, 1974). Recently, Hughes & Roth (1974) reported that the release of exogenously incorporated noradrenaline in the rabbit portal vein is dependent on stimulus train length while the release of newly synthesized noradrenaline is not. This study was carried out to see if the potentiation of the response of the portal vein to electrical stimulation was dependent on stimulus train length.

Methods Male albino rabbits (1.5-3 kg) were killed by a blow on the head. The portal vein was dissected out as described by Hughes & Vane (1967). Each vein was suspended in an organ bath containing 15 ml of Krebs-Henseleit solution (mM: NaCl 118.0, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.1, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 11.0) kept at 37.5°C and bubbled with a gas mixture of 95% O_2 and 5% CO_2. A tension of 3 g was applied to the veins which were then allowed to equilibrate for 1 h before they were stimulated electrically. Isometric contractions were measured with Grass FTO3 force displacement transducers and recorded on a model 7 Grass polygraph. A Grass S4 stimulator was used for field stimulation; a current was applied between 2 platinum electrodes, one placed at each side of the organ bath. The veins were stimulated at frequencies of 2 or 5 Hz, with a pulse duration of 1 ms at supramaximal voltage. Indomethacin was dissolved in a 2% sodium carbonate solution and the pH adjusted to 7.6 with HCl. The indomethacin concentration is expressed as final bath concentration of the free base and added to the bath in a volume of 0.1 ml.

Results Eight experiments were done to determine the effect of train length on the potentiation of the contractile response of the portal vein to electrical stimulation by indomethacin. Four veins were stimulated at a frequency of 2 Hz with trains of 30, 60, 120 and 240 pulses, and four veins at 5 Hz with trains of 75, 150, 300 and 600 pulses in the absence and presence of indomethacin (10 μg/ml). The veins were allowed to relax for 15 min between each period of stimulation. Indomethacin caused a significant potentiation of the responses to 2 Hz at all train lengths but significantly reduced the responses to 5 Hz at all train lengths. The results are summarized in Table 1. In eight additional veins there was no significant difference in the response obtained to repeated electrical stimulation at 2 Hz or 5 Hz

Table 1 The effect of indomethacin (10 μg/ml) on the contractile response of the portal vein of the rabbit to electrical stimulation

	Tension (g)	
No. of pulses per train	*Control response*	*Change in response after indomethacin*
	Frequency 2 Hz	
30	0.80 ± 0.17	+0.68 ± 0.07***
60	1.20 ± 0.19	+0.98 ± 0.22**
120	1.48 ± 0.22	+1.41 ± 0.41*
240	1.63 ± 0.26	+2.05 ± 0.41**
	Frequency 5 Hz	
75	4.10 ± 0.45	−0.20 ± 0.06*
150	5.15 ± 0.43	−0.55 ± 0.18*
300	5.90 ± 0.46	−0.45 ± 0.16*
600	6.15 ± 0.44	−0.43 ± 0.15*

Figures shown are the mean of 4 experiments with the s.e.mean. Significance of difference from controls (*t* test for paired data): * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

with any of the train lengths in the absence of any drug treatment.

Discussion The exact role of prostaglandins in sympathetic neurotransmission is not completely clear. However, it is well established that prostaglandins of the E type inhibit the release of noradrenaline in response to sympathetic nerve stimulation and that inhibition of prostaglandin synthesis with indomethacin facilitates the release of noradrenaline. On the basis of this evidence it has been postulated that the prostaglandins of the E type play a regulatory role in sympathetic neurotransmission (Hedqvist, 1973). The responses of the isolated portal vein of the rabbit to electrical stimulation are inhibited by prostaglandin E_2 and potentiated by indomethacin. Both the inhibition and potentiation are frequency-dependent. Inhibition of noradrenaline synthesis with α-methyl-*p*-tyrosine abolishes this indomethacin potentiation. These results suggested that the noradrenaline which is released at low frequencies is newly synthesized and that endogenous prostaglandins control this release (Greenberg, 1974).

It has been suggested by Hughes & Roth (1972) that in the portal vein and vas deferens of the rabbit, noradrenaline may be released from different pools at different frequencies of stimulation. They further reported that the release of exogenously incorporated noradrenaline was influenced by the number of pulses in a train at a given frequency while the release of newly synthesized noradrenaline was not (Hughes & Roth, 1974).

The results described here show that the potentiation by indomethacin of the responses of the rabbit portal vein to electrical stimulation are dependent on frequency but not on the number of pulses in a train. These results are therefore consistent with the lack of effect of train length on the release of newly synthesized noradrenaline, and add further support for the idea that newly synthesized noradrenaline is released at low frequencies and that this release is influenced by prostaglandins of the E type. However, confirmation of this point of view can only be made by the simultaneous measurement of exogenously incorporated and newly synthesized noradrenaline released on nerve stimulation.

I wish to acknowledge the competent technical assistance of Mr G. Levesque.

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EFFECT OF TETRAMETHYLENEDISULPHOTETRAMINE ON THE MEMBRANE CONDUCTANCE INCREASE PRODUCED BY γ-AMINO-BUTYRIC ACID AT THE CRAB NEUROMUSCULAR JUNCTION

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The effect of tetramethylenedisulphotetramine (TETS) on the increase in membrane conductance produced by γ-aminobutyric acid (GABA) at the hermit crab neuromuscular junction was investigated. TETS produced a dose-dependent antagonism of GABA, which was characterized by a non-parallel shift of the log dose-conductance curve for GABA with a reduced maximal conductance change.

Introduction In the preceding paper Bowery, Brown & Collins (1975) showed that tetramethylenedisulphotetramine (TETS) antagonized the depolarizing action of γ-aminobutyric acid (GABA) on rat sympathetic ganglia, in an apparently non-competitive manner. The present paper describes the action of TETS on the responses of crustacean muscle to GABA. GABA is probably the inhibitory transmitter at crustacean neuromuscular junctions and produces an increased membrane conductance.

Methods Conductance changes produced by GABA in the abductor muscle of large claws of the hermit crab (*Eupagurus bernhardus*) were measured as described previously (Earl & Large, 1974). Conductance measurements were made 1 min after the addition of each concentration of GABA. TETS was dissolved in acetone at 10 mg ml^{-1} and an appropriate dilution in Ringer solution added 2 min before GABA. Control solutions contained an equivalent amount of acetone. The composition of the Ringer solution was (mM): NaCl, 445; KCl, 12.2; $CaCl_2$, 29.6, $MgCl_2$, 5.75 and $NaHCO_3$, 1.79. TETS was synthesized by J.F. Collins.

Results At concentrations of 20 μM upwards TETS consistently reduced the conductance change produced by GABA (9 experiments). The log dose-conductance curve for GABA showed a parallel shift to the right at low TETS concentration and a reduced maximum response at high concentrations (Figure 1). TETS was rapidly washed out, usually within the time required to wash out the GABA (10-20 minutes).

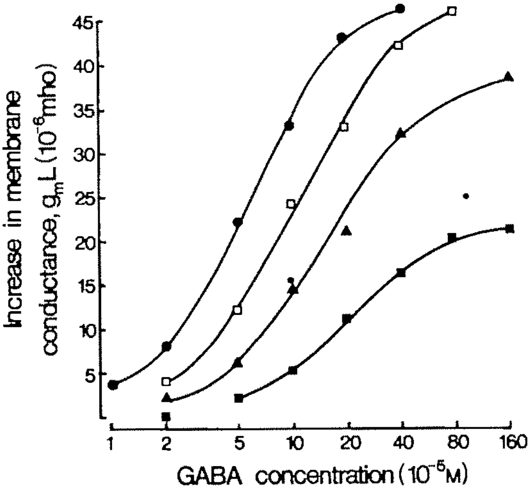

Figure 1 Effect of tetramethylenedisulphotetramine (TETS) on the relation between membrane conductance $g_m L$ (where g_m is the membrane conductance of 1 cm of muscle fibre and L is the half-length of the fibre) and the concentration of γ-aminobutyric acid (GABA). (●) Control; (□), 2×10^{-5} M TETS; (▲), 5×10^{-5} M TETS; (■), 1×10^{-4} M TETS (these results obtained from a single cell).

TETS alone did not alter membrane conductance and did not reduce the amplitude of excitatory junction potentials beyond that produced by the equivalent amount of acetone (6 experiments).

Discussion The present experiments confirm the antagonism of TETS to GABA previously observed in ganglia (Bowery *et al.*, 1975). Antagonism does not appear to derive from a non-specific effect on membrane conductance and probably does not extend to glutamate since the excitatory junction potentials were not depressed (glutamate is probably the excitatory transmitter at the crustacean neuromuscular junction, see Gerschenfeld, 1973).

The antagonism of GABA by TETS appears to have the same characteristics already observed with picrotoxin and bicuculline at this synapse (Earl & Large, 1974); namely, the antagonists reduce the maximal response of GABA and hence suggest a non-competitive type of antagonism. However it is interesting to note that, in low concentrations, TETS produces a parallel shift of the GABA dose-response curve, as is found with picrotoxin (unpublished observation, Earl & Large).

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As judged from the depression in maximal conductance change, TETS appears to be about 10 times more potent than bicuculline but 10 times less potent than picrotoxin (i.e. the order of potency is bicuculline < TETS < picrotoxin). In the ganglion the order of increasing potency is approximately picrotoxin < bicuculline ≈ TETS (Bowery *et al.*, 1975). This may reflect chemical differences between crustacean and mammalian GABA receptors (cf. Bowery & Brown, 1974).

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INDEX TO VOLUME 53

AUTHOR INDEX

A

ADAMS, P.R. Kinetics of agonist conductance changes during hyperpolarization at frog endplates, 308

ADAMS, P.R. & BROWN, D.A. Actions of γ-aminobutyric acid on ganglionic transmission and ganglion cell excitability, 438P

ADLER-GRASCHINSKY, EDDA & LANGER, S.Z. Possible role of a β-adrenoceptor in the regulation of noradrenaline release by nerve stimulation through a positive feed-back mechanism, 43

AHTEE, LIISA, Effect of drugs on metoclopramide-induced catalepsy and increase in striatal homovanillic acid content, 460P

ANLEZARK, G.M. & MELDRUM, B.S. Effects of apomorphine, ergocornine and piribedil on audiogenic seizures in DBA/2 mice, 419

ATKINSON, D.C. & HICKS, R. Possible occurrence of endogenous anti-inflammatory substances in blood of injured rats, 85

ATTERWILL, C.K., MAHONEY, ANNE & NEAL, M.J. Uptake and subcellular distribution of ^{3}H-choline by retina, 447P

AUNIS, D., MANDEL, P., MIRAS-PORTUGAL, M.T., COQUILLAT, G., ROHMER, F. & WARTER, J.M. Changes of human plasma dopamine-β-hydroxylase activity after intravenous administration of theophylline, 425

AUTY, R.M. & BRANCH, R.A. (introduced by BHOOLA, K.D.). Metabolism of ethyl, *n*-propyl, *n*-butyl and *iso*-amyl alcohols by isolated rat liver, 443P

el-AZHARY, ROKEA A., KHAYYAL, M.T. & SALEH, SAMIRA. Effect of fluphenazine on tissue noradrenaline concentrations and its interaction with paragyline, 593

B

BARLOW, R.B. Use of logistic function for calculation of dose-ratios and potency ratios, 139

BEART, P.M. *see* MOORE, K.E., 453P

BEDWANI, J.R. & MARLEY, P.B. Enhanced inactivation of prostaglandin E$_2$ by the rabbit lung during pregnancy or progesterone treatment, 547

BENNETT, T. & GARDINER, SHEILA M. (introduced by BIRMINGHAM, A.T.). Effects of intravenous injections of vinblastine or vincristine on responses of rat heart to nerve stimulation and to drugs, 444P

BENOY, CHRISTINE J., FELLAH, M.S. EL, SCHNEIDER, R. & WADE, O.L. Tolerance to sympathomimetic bronchodilators in the guinea-pig lungs, 463P

BERDE, B. *see* MEIER, J., 440P

BEVAN, P., BRADSHAW, C.M. & SZABADI, E. The effect of tricyclic antidepressants on cholinergic responses of single cortical neurones, 29

BEVAN, P., BRADSHAW, C.M. & SZABADI, E. Tricyclic antidepressants and monoamines: the relationship between uptake blockade and potentiation of neuronal responses, 459P

BHOOLA, K.D. *see* AUTY, R.M., 443P

BIRMINGHAM, A.T. *see* BENNETT, T., 444P

BLACK, J.W. *see* BRIMBLECOMBE, R.W., 435P

BLACKWELL, G.J., FLOWER, R.J., PARSONS, M.F. & VANE, J.R. Factors influencing the turnover of prostaglandin synthetase, 467P

BLOGG, C.E., BRITTAIN, R.T., SIMPSON, B.R. & TYERS, M.B. AH 10407: a novel short-acting competitive neuromuscular blocking drug in animals and man, 446P

BOND, P.A., BROOKS, B.A. & JUDD, A. The distribution of lithium, sodium and magnesium in rat brain and plasma after various periods of administration of lithium in the diet, 235

BOTTING, J.H. Sensitivity of neonatal rabbit ileum to histamine, 428

BOULLIN, D.J., GRAHAME-SMITH, D.G., GRIMES, R.P.J. & WOODS, H.F. Inhibition of 5-hydroxytryptamine-induced human blood platelet aggregation by chlorpromazine and its metabolites, 121

BOWERY, N.G., BROWN, D.A. & COLLINS, J.F. Tetramethylenedisulphotetramine: an inhibitor of γ-aminobutyric acid induced depolarization of the isolated superior cervical ganglion of the rat, 422

BRADLEY, P.B. & BRAMWELL, G.J. A stereospecific action of morphine on brain stem neuronal activity: a microiontophoretic study, 462P

BRADSHAW, C.M. *see* BEVAN, P., 29

BRADSHAW, C.M. *see* BEVAN, P., 459P

BRAMWELL, G.J. *see* BRADLEY, P.B., 462P

BRANCH, R.A. *see* AUTY, R.M., 443P

BRIMBLECOMBE, R.W., DUNCAN, W.A.M., DURANT, G.J., GANELLIN, C.R., PARSONS, M.E. & BLACK, J.W. Pharmacology of cimetidine, a new histamine H$_2$-receptor antagonist, 435P

BRIMBLECOMBE, R.W., BUXTON, D.A. & REDFERN, P.H. Drug effects on avoidance behaviour in selected strains of rats, 461P

BRITTAIN, R.T. *see* BLOGG, C.E., 446P

BROOKS, B.A. *see* BOND, P.A., 235

BROWN, D.A. *see* ADAMS, P.R., 438P

BROWN, D.A. *see* BOWERY, N.G., 422

BRYANT, B.J., McCULLOCH, M.W., RAND, M.J. & STORY, D.F. Release of ^{3}H-(–)-noradrenaline from guinea-pig hypothalamic slices: effects of adrenoceptor agonists and antagonists, 454P

BUCZKO, W., GAETANO, G. DE & GARATTINI, S. Effect of fenfluramine on 5-hydroxytryptamine uptake and release by rat blood platelets, 563
BURDEN, D.T. & PARKES, M.W. An investigation of the tachycardia produced by i.c.v. injections of isoprenaline in mice, 341
BUXTON, D.A. *see* BRIMBLECOMBE, R.W., 461P

C

CALLINGHAM, B.A. & LYLES, G.A. Some effects of age upon irreversible inhibition of cardiac MAO, 458P
CHAMALES, M.H., GOURLEY, R.D. & WILLIAMS, BETTY J. Effect of acetylcholine on changes in contractility, heart rate and phosphorylase activity produced by isoprenaline, salbutamol and aminophylline in perfused guinea-pig hearts, 531
CHAWLA, NARESH, JOHRI, M. B. L., SAXENA, P.N. & SINGHAL, K.C. Cholinergic mechanisms in central thermoregulation in pigeons, 317
CHIOU, C.Y. & MALAGODI, MARJORIE H. Studies on mechanism of action of a new Ca^{2+} antagonist, 8-(*N,N*-diethylamino)octyl 3, 4, 5-trimethoxybenzoate hydrochloride, in smooth and skeletal muscles, 279
COLLINS, J.F. *see* BOWERY, N.G., 422
CONSTANTI, A. & NISTRI, A. (introduced by QUILLIAM, J.P.). Actions of glutamate and kainic acid on lobster muscle fibre and frog spinal cord, 437P
COQUILLAT, G., *see* AUNIS, D., 425
COTTNEY, J. & LEWIS, A.J. Mechanisms of concanavalin A-induced inflammation in rat, 445P
COX, B.M., GINSBURG, M. & WILLIS, JULIA (with Appendix by DAVIES, J.K.). The offset of morphine tolerance in rats and mice, 383
CROCKER, ANN D. & WILSON, K.A. A further investigation into the energy dependence of angiotensin II-induced contractions of isolated smooth muscle preparations, 59
CRUTCHLEY, D.J. & PIPER, PRISCILLA J. Inhibition of the pulmonary inactivation of prostaglandins in rabbit *in vivo*, 467P
CURWAIN, B.P. & MILLS, G.A. Effects of atropine on gastric secretion and mucosal blood flow in conscious dog, 432P

D

DA PRADA, M., OBRIST, R. & PLETSCHER, A. Discrimination of monoamine uptake by membranes of adrenal chromaffin granules, 257
DALY, M.J., FLOOK, JUDITH J. & LEVY, G.P. The selectivity of β-adrenoceptor antagonists on cardiovascular and bronchodilator responses to isoprenaline in the anaesthetized dog, 173
DARGIE, H.J., DOLLERY, C.T. & LEWIS, P.J. Prevention of DOCA saline hypertension by central 6-hydroxydopamine; role of saline intake, 455P
DAVIES, J.K. *see* COX, B.M., 383
DAWES, P.M., *see* FAULKNER, D.C. Effect of propranolol on vascular responses to sympathetic nerve stimulation, 517
DE MICHELI, P. & GLASSER, A.H. Effects of catecholamines and adrenoceptor blocking drugs on canine peripheral lymph flow, 499
DICK, FABIENNE & KELLY, J.S. L-2,4-diaminobutyric acid as a selective marker for inhibitory nerve terminals in rat brain, 439P
DOLLERY, C.T. *see* DARGIE, H.J., 455P
DONOVAN, B.T. Indomethacin, ketoprofen and corpus luteum regression in the guinea-pig, 225
DUNCAN, W.A.M. *see* BRIMBLECOMBE, R.W., 435P
DURANT, G.J. *see* BRIMBLECOMBE, R.W., 435P

E

ELKHAWAD, A.O., MUNDAY, K.A., POAT, JUDITH A. & WOODRUFF, G.N. The effect of dopamine receptor stimulants on locomotor activity and cyclic AMP levels in the rat striatum, 456P
ELLIOTT, ANNE B. Effect of oxytocin on blood vessels of cremaster muscle of rat, 149
ENERO, MARIA A. & LANGER, S.Z. Pharmacological effects of histamine on isolated cat nictitating membrane, 431
EVANS, D.P. & THOMSON, D.S. Inhibition of immediate hypersensitivity reactions in laboratory animals by a phenanthroline salt (ICI 74,917), 409

F

FANTOZZI, R., FRANCONI, FLAVIA, MANNAIONI, P.F., MASINI, EMANUELA & MORONI, F. Interaction of histamine H_1- and H_2-receptor antagonists with histamine uptake and metabolism by guinea-pig isolated atrium and mouse neoplastic mast-cells *in vitro*, 569
FAULKNER, D.C. *see* DAWES, P.M., 517
FELLAH, M.S. EL *see* BENOY, CHRISTINE J., 463P
FLOOK, JUDITH J. *see* DALY, M.J., 173
FLOWER, R.J. *see* BLACKWELL, G.J., 467P
FOSTER, R.W. & O'DONNELL, STELLA R. Evidence that adrenergic nerves are responsible for active uptake of noradrenaline in guinea-pig isolated trachea, 109
FOSTER, R.W. & RAMAGE, A.G. Observations on effect of dienzoxazepine and *N*-nonyl-vanillylamide on sensory nerves, 436P
FOZARD, J.R. & MWALUKO, G.M.P. Stimulant effects of 5-hydroxytryptamine on cardiac sympathetic nerves, 449P
FRANCONI, FLAVIA *see* FANTOZZI, R., 569

G

GAETANO, G. DE *see* BUCZKO, W., 563
GLASSER, A.H. *see* DE MICHELI, P., 499
GANELLIN, C.R. *see* BRIMBLECOMBE, R.W., 435P
GARATTINI, S. *see* BUCZKO, W., 563
GARCIA, A.G. & SANCHEZ-GARCIA, P. Influence of cocaine and sodium on bretylium uptake by reserpine-treated guinea-pig left atrium, 247
GARDINER, SHEILA M. *see* BENNETT, T., 444P
GINSBURG, M. *see* COX, B.M., 383
GOURLEY, R.D. *see* CHAMALES, M.H., 531
GRAHAM, C.W., GREEN, A.R., WOODS, H.F. & YOUDIM, M.B.H. 5-Hydroxytryptamine synthesis in isolated perfused rat brain, 449P
GRAHAME-SMITH, D.G. *see* BOULLIN, D.J., 121

GRAYSON, J. & SCOTT, C. The action of nitroglycerine and dipyridamole in normal and ischaemic dog heart, 11

GREAVES, M.W., KINGSTON, W.P. & PRETTY, KATHLEEN. Action of a series of non-steroid and steroid anti-inflammatory drugs on prostaglandin synthesis by the microsomal fraction of rat skin, 470P

GREEN, A.R. *see* GRAHAM, C.W., 450P

GREEN, A.R., SOURKES, T.L. & YOUNG, S.N. Liver and brain tryptophan metabolism following hydrocortisone administration to rats and gerbils, 287

GREENBERG, R. Indomethacin potentiation of the response of the rabbit portal vein to electrical stimulation: effect of stimulus frequency and train length, 596

GREENWOOD, B. & KERRY, P.J. Prostaglandin production by a mild inflammatory lesion in sheep, 305

GRIMES, R.P.J. *see* BOULLINE, D.J., 121

GULATI, O.D. & SIVARAMAKRISHNA, N. Kinetics and some characteristics of uptake of noradrenaline by human umbilical artery, 152

H

HAKANSON, R., HEDENBRO, J., LIEDBERG, G., REHFELD, J.F. & STADIL, F. Activation of histamine decarboxylase by H_2-receptor blockade. mechanism of action, 127

HARRIS, D.W., SMY, J.R., REED, J.D. & VENABLES, C.W. Effects of burimamide and metiamide on basal gastric function in cat, 293

HAYASHI, E. & YAMADA, S. Pharmacological studies on surugatoxin, the toxic principle from Japanese ivory mollusc (*Babylonia japonica*), 207

HEDENBRO, J. *see* HAKANSON, R., 127

HENDERSON, G., HUGHES, J. & KOSTERLITZ, H.W. Effects of morphine on release of noradrenaline from cat isolated nictitating membrane and guinea-pig ileum myenteric plexus-longitudinal muscle preparation, 505

HERMAN, A.G. & MONCADA, S. Release of prostaglandins and incapacitation after injection of endotoxin in the knee joint of the dog, 465P

HEWICK, D.S. & MORELAND, T.A. (introduced by MARSHALL, P.B.). An NADPH-dependent warfarin reductase in human and rat liver and kidney soluble fraction, 441P

HICKS, R. *see* ATKINSON, D.C., 85

HIGGS, G.A., McCALL, ELAINE & YOULTEN, L.J.F. A chemotactic role for prostaglandins released from polymorphonuclear leucocytes during phagocytosis, 539

HIRSCH, J.C., TASSIN, J.P. & THIERRY, A.M. Loss of noradrenergic terminals in the chronically isolated cerebral cortex of the cat, 455P

HODGES, J.R. & VELLUCCI, SANDRA V. The effect of reserpine on hypothalamo-pituitary-adrenocortical function in the rat, 555

HOLMAN, R.B., HOYLAND, VALERIE & SHILLITO, ELIZABETH E. Failure of *p*-chlorophenylalanine to affect voluntary alcohol consumption in rats, 299

HOLTON, PAMELA *see* McISAAC, R.L., 434P

HOLTON, PAMELA, IMPICCIATORE, MARIANINA & SPENCER, JAN. Dose response relationship and comparison of secretory potency of methyl histamine and histamine on isolated guinea-pig stomach, 432P

HOOD, A.J.C., SMY, J.R. & WEETMAN, D.F. An indirect sympathomimetic effect of burimamide on kitten isolated atria, 525

HOYLAND, VALERIE *see* HOLMAN, R.B., 299

HUGHES, J. *see* HENDERSON, G., 505

HUGHES, J., KOSTERLITZ, H.W. & LESLIE, FRANCES M. Effect of morphine on adrenergic transmission in the mouse vas deferens. Assessment of agonist and antagonist potencies of narcotic analgesics, 371

I

IMPICCIATORE, MARIANINA *see* HOLTON, PAMELA, 432P

IVERSEN, L.L. *see* MILLER, R., 457P

J

JOHNS, E.J., RICHARDS, H.K. & SINGER, BERTHA. Effects of adrenaline, noradrenaline, isoprenaline and salbutamol on the production and release of renin by isolated renal cortical cells of the cat, 67

JOHNSON, K.G. Thermoregulatory change induced by cholinomimetic substances introduced into the cerebral ventricles of sheep, 489

JOHRI, M.B.L. *see* CHAWLA, NARESH, 317

JONES, MARGARET E.L. & SPRIGGS, T.L.B. Noradrenaline and motor transmission in vas deferens of mouse, 323

JONES, R.L. Actions of prostaglandins on the arterial system of the sheep: some structure-activity relationships, 464P

JUDD, A. *see* BOND, P.A., 235

K

KALSNER, S. Role of extraneuronal mechanisms in the termination of contractile responses to amines in vascular tissue, 267

KELLY, J.G. & SHANKS, R.G. Metabolic and cardiovascular effects of isoprenaline and salbutamol in the dog, 157

KELLY, J.S. *see* DICK, FABIENNE, 439P

KENNEDY, I. & LEVY, G.P. Combined α- and β-adrenoceptor blocking drug AH 5158: further studies on α-adrenoceptor blockade in anaesthetized animals, 585

KERRY, P.J. *see* GREENWOOD, B., 305

KHAYYAL, M.T. *see* el-AZHARY, ROKEA A., 593

KINGSTON, W.P. *see* GREAVES, M.W., 470P

KIRSTEN, VAAJE *see* NUSTAD, K., 229

KOSTERLITZ, H.W. *see* HENDERSON, G., 505

KOSTERLITZ, H.W. *see* HUGHES, J., 371

KOSTERLITZ, H.W. *see* HUGHES, J., 505

KOSTERLITZ, H.W. & WATERFIELD, ANGELA A. Analysis of phenomenon of acute tolerance to morphine in guinea-pig, isolated ileum, 131

KRAFT, E. & ZIMMERMAN, B.G. Influence of histamine H_1- and H_2-receptor blockers on sympathetic vasodilator and vasoconstrictor responses in canine paw, 51

L

LANGER, S.Z. *see* ADLER-GRASCHINSKY, EDDA, 43
LANGER, S.Z. *see* ENERO, MARIA A., 431P
LARGE, W.A. Effect of tetramethylenedisulphotetramine on the membrane conductance increase produced by γ-aminobutyric acid at the crab neuromuscular junction, 598
LEME, J. GARCIA & SCHAPOVAL, ELFRIDGES E.S. Stimulation of the hypothalamo-pituitary-adrenal axis by compounds formed in inflamed tissue, 75
LESLIE, FRANCES M. *see* HUGHES, J., 371
LEVY, G.P. *see* DALY, M.J., 173
LEVY, G.P. *see* KENNEDY, I., 585
LEWIS, A.J. *see* COTTNEY, J., 445P
LEWIS, P.J. *see* DARGIE, H.J., 455P
LIVINGSTONE, A. & PHILLIPS, ELIZABETH. Distribution of chlorpromazine and its metabolites in subfraction of rat brain, 451P
LIEDBERG, G. *see* HAKANSON, R., 127
LOVELESS, A.H. & MAXWELL, D.R. Prevention by sulphydril compounds of toxicity in cat of 2,6-dimethoxyphenol and its morpholinepropionyl ester, 93
LÜLLMANN, H., PETERS, T. & RAVENS, URSULA. Studies on kinetics of [^{3}H]-ouabain uptake and exchange in isolated papillary muscle of guinea-pig, 99
LYLES, G.A. *see* CALLINGHAM, B.A., 458P

M

MAHONEY, ANNE *see* ATTERWILL, C.K., 447P
MALAGODI, MARJORIE H. *see* CHIOU, C.Y., 279
MARSHALL, P.B. *see* HEWICK, D.S., 441P
MAXWELL, D.R. *see* LOVELESS, A.H., 93
McCALDEN, T.A. The inhibitory action of oestradiol-17-β and progesterone on venous smooth muscle, 183
McCALL, ELAINE *see* HIGGS, G.A., 539
McCULLOCH, M.W. *see* BRYANT, B.J., 454P
McISAAC, R.L. & WILSON, G.M. (introduced by HOLTON, PAMELA). Effect of atropine on pentagastrin-induced gastric acid secretion and mucosal blood flow in conscious rat, 434P
McKILLOP, D. & POWIS, G. Effect of a single dose of ethanol on hepatic microsomal metabolism of foreign compounds in rat, 442P
MAIN, I.H.M. & WHITTLE, B.J.R. Investigation of the vasodilator and anti-secretory role of prostaglandins in the rat gastric mucosa by use of non-steroidal anti-inflammatory drugs, 217
MANDEL, P. *see* AUNIS, D., 425
MANNAIONI, P.F. *see* FANTOZZI, R., 569
MARLEY, E. & WHELAN, JENNIFER E. Some central effects of 5-hydroxytryptamine in young chickens at and below thermoneutrality, 37
MARLEY, P.B. *see* BEDWANI, J.R., 547
MARLEY, E. & NISTICO, G. Tryptamines and some other substances affecting waking and sleep in fowl, 193
MASINI, EMANUELA *see* FANTOZZI, R., 569
MEIER, J. (introduced by BERDE, B.). Bioanalytical assay of clozapine and its *N*-oxide metabolite and determination of blood levels in dog, 440P
MELDRUM, B.S. *see* ANLEZARK, G.M., 419
METCALF, G. & THOMPSON, J.W. The effect of various amine-depleting drugs on the fever response exhibited by rabbits to bacterial or leucocyte pyrogen, 21
MILLER, R. (introduced by IVERSEN, L.L.). Inhibition of dopamine-sensitive adenylate cyclase in rat basal ganglia and other hormone sensitive adenylate cyclase systems by neuroleptic drugs, 457P
MILLS, G.A. *see* CURWAIN, B.P., 432P
MINATOYA, H. & SPILKER, B.A. Lack of cardiac or bronchodilator tachyphylaxis to isoprenaline in dog, 333
MIRAS-PORTUGAL, M.T. *see* AUNIS, D., 425
MOORE, K.E. & PHILLIPSON, O.T. (introduced by BEART, P.M.). Effects of dexamethasone on phenylethanolamine *N*-methyl-transferase (PMNT) and adrenaline (A) in the brains of adult and neonatal rats, 453P
MONCADA, S. *see* HERMAN, A.G., 465P
MORELAND, T.A. *see* HEWICK, D.S., 441P
MORONI, F. *see* FANTOZZI, R., 569
MUNDAY, K.A. *see* ELKHAWAD, A.O., 456P
MURNAGHAN, M.F. The effect of sympathomimetic amines on the ventricular fibrillation threshold in the rabbit isolated heart, 3
MWALUKO, G.M.P. *see* FOZARD, J.R., 449P

N

NEAL, M.J. *see* ATTERWILL, ANNE, 447P
NESHEIM, BRITT-INGJERD. Action of β-adrenoceptor antagonists on the response to isoprenaline in the oestrogen dominated rabbit uterus, 393
NESHEIM, BRITT-INGJERD, OSNES, J.-B. & ØYE, I. Role of cyclic adenosine 3′,5′-monophosphate in the isoprenaline-induced relaxation of the oestrogen dominated rabbit uterus, 403
NISTICO, G. *see* MARLEY, E., 193
NISTRI, A. *see* CONSTANTI, A., 437P
NORTHOVER, B.J. Effect of anti-inflammatory drugs on membrane potential of vascular endothelial cells *in vitro*, 113
NUSTAD, K., PIERCE, J.V. & KIRSTEN, VAAJE. Synthesis of kallikreins by rat kidney slices, 229

O

OBRIST, R. *see* DA PRADA, M., 257
O'DONNELL, STELLA R. *see* FOSTER, R.W., 109
OSNES, J.-B. *see* NESHEIM, BRITT-INGJERD, 403
ØYE, I. *see* NESHEIM, BRITT-INGJERD, 403

P

PACHA, W., SALZMANN, R. & SCHOLTYSIK, G. Inhibitory effects of clonidine and BS 100-141 on responses to sympathetic nerve stimulation in cats and rabbits, 513
PAOLONI, H.J. & WILCKEN, D.E.L. The action of aminophylline on the acutely transplanted dog heart: effect of α- and β-adrenoceptor blockade, 163
PARKES, M.W. *see* BURDEN, D.T., 341
PARRATT, J.R. & STURGESS, R.M. *E. coli* endotoxin shock in cat: treatment with indomethacin, 485
PARRATT, J.R. & STURGESS, R.M. The protective

effect of sodium meclofenamate in experimental endotoxin shock, 466P
PARSONS, M.E. *see* BRIMBLECOMBE, R.W., 435P
PARSONS, M.F. *see* BLACKWELL, G.J., 467P
PARVEZ, SIMONE, PARVEZ, S.H. & YOUDIM, M.B.H. Variation in activity of monoamine-metabolizing enzymes in rat liver during pregnancy, 241
PARVEZ, S.H. *see* PARVEZ, SIMONE, 241
PEARSON, J.D.M. & SHARMAN, D.F. Estimation of 3,4-dihydroxyphenylacetic acid, homovanillic acid and homo-isovanillic acid in nervous tissue by gas-liquid chromatography and electron capture detection, 143
PETERS, T. *see* LÜLLMANN, H., 99
PHILLIPS, ELIZABETH *see* LIVINGSTONE, A., 451P
PHILLIPSON, O.T. *see* MOORE, K.E., 453P
PIERCE, J.V. *see* NUSTAD, K., 229
PIPER, PRISCILLA J. *see* CRUTCHLEY, D.J., 467P
PLETSCHER, A. *see* DA PRADA, M., 257
POAT, JUDITH A. *see* ELKHAWAD, A.O., 456P
POWIS, G. *see* McKILLOP, D., 442P
PRETTY, KATHLEEN *see* GREAVES, M.W., 470P
PUGH, D.M., SHARMA, S.C. & WILSON, C.W.M. Inhibitory effect of L-ascorbic acid on the yield of prostaglandin F from the guinea-pig uterine homogenates, 469P

Q

QUILLIAM, J.P. *see* CONSTANTI, A., 437P

R

RAMAGE, A.G. *see* FOSTER, R.W., 436P
RAND, M.J. *see* BRYANT, B.J., 454P
RAVENS, URSULA *see* LULLMANN, H., 99
REDFERN, P.H. *see* BRIMBLECOMBE, R.W., 461P
REED, J.D. *see* HARRIS, D.W., 293
REHFELD, J.F. *see* HAKANSON, R., 127
RICHARDS, H.K. *see* JOHNS, E.J., 67
ROBINSON, D.A., TAYLOR, J.G. & YOUNG, J.M. The irreversible binding of acetylcholine mustard to muscarinic receptors in intestinal smooth muscle of the guinea-pig, 363
ROHMER, F. *see* AUNIS, D., 425
RUBIN, R.P. & WARNER, W. Nicotine-induced stimulation of steroidogenesis in adrenocortical cells of cat, 357

S

SALEH, SAMIRA *see* el-AZHARY, ROKEA A., 593
SALZMANN, R. *see* PACHA, W., 513
SANCHEZ-GARCIA, P. *see* GARCIA, A.G., 247
SAXENA, P.N. *see* CHAWLA, NARESH, 317
SCHAPOVAL, ELFRIDES E.S. *see* LEME, J. GARCIA, 75
SCHILD, H.O. An ambiguity in receptor theory, 311
SCHILD, H.O. Drug desensitization and cross-desensitization in guinea-pig ileum, 448P
SCHNEIDER, R. *see* BENOY, CHRISTINE J., 463P
SCHOLTYSIK, G. *see* PACHA, W., 513
SCOTT, C. *see* GRAYSON, J., 11
SHANKS, R.G. *see* KELLY, J.G., 157
SHARMA, S.C. *see* PUGH, D.M., 469P
SHILLITO, ELIZABETH E. *see* HOLMAN, R.B., 299
SIMPSON, B.R. *see* BLOGG, C.E., 446P
SINGER, BERTHA *see* JOHNS, E.J., 67
SINGHAL, K.C. *see* CHAWLA, NARESH, 317
SIVARAMAKRISHNA, N. *see* GULATI, O.D., 152
SLOTKIN, T.A. Effects of reserpine on the adrenal medulla of the spontaneously hypertensive rat, 349
SMY, J.R. *see* HARRIS, D.W., 293
SMY, J.R. *see* HOOD, A.J.C., 525
SNYDER, S.H. (In collaboration with YOUNG, ANNE B.). Fifth Gaddum Memorial Lecture–Glycine synaptic receptor in mammalian CNS, 473
SOURKES, T.L. *see* GREEN, A.R., 287
SPEDDING, M., SWEETMAN, A.J. & WEETMAN, D.F. Antagonism of adenosine 5′-triphosphate-induced relaxation of 2-2′-pyridylisatogen in the taenia of guinea-pig caecum, 575
SPENCER, JAN *see* HOLTON, PAMELA, 432P
SPILKER, B.A. *see* MINATOYA, H., 333
SPRIGGS, T.L.B. *see* JONES, MARGARET E.L., 323
STADIL, F. *see* HAKANSON, R., 127
STORY, D.F., *see* BRYANT, B.J., 454P
STURGESS, R.M. *see* PARRATT, J.R., 466P
STURGESS, R.M. *see* PARRATT, J.R., 485
SWEETMAN, A.J. *see* SPEDDING, M., 575
SZABADI, E. *see* BEVAN, P., 29
SZABADI, E. *see* BEVAN, P., 459P

T

TASSIN, J.P. *see* HIRSCH, J.C., 455P
TAYLOR, J.G. *see* ROBINSON, D.A., 363
THIERRY, A.M., *see* HIRSCH, J.C., 455P
THOMPSON, J.W. *see* METCALF, G., 21
THOMSON, D.S. *see* EVANS, D.P., 409
TRIST, D.G. & WEATHERALL, M. Effect of isoprenaline on ^{86}Rb uptake by horse lymphocytes *in vitro*, 448P
TURNBULL, M.J. & WATKINS, J.W. Change in sensitivity to pentobarbitone and halothane induced by acute administration of central nervous system depressant drugs, 452P
TYERS, M.B. *see* BLOGG, C.E., 446P

V

VANE, J.R. *see* BLACKWELL, G.J., 467P
VELLUCCI, SANDRA V. *see* HODGES, J.R., 555
VENABLES, C.W. *see* HARRIS, D.W., 293

W

WADE, O.L. *see* BENOY, CHRISTINE J., 463P
WARNER, W. *see* RUBIN, R.P., 357
WARTER, J.M. *see* AUNIS, D., 425
WATERFIELD, ANGELA A. *see* KOSTERLITZ, H.W., 131
WATKINS, J.W. *see* TURNBULL, M.J., 452P
WEATHERALL, M. *see* TRIST, D.G., 448P
WEETMAN, D.F. *see* HOOD, A.J.C., 525
WEETMAN, D.F. *see* SPEDDING, M., 575
WHELAN, JENNIFER E. *see* MARLEY, E., 37
WHITTLE, B.J.R. *see* MAIN, I.H.M., 217
WILCKEN, D.E.L. *see* PAOLONI, H.J., 163
WILLIAMS, BETTY J. *see* CHAMALES, M.H., 531

WILLIS, JULIA see COX, B.M., 383
WILSON, C.W.M. *see* PUGH, D.M., 469P
WILSON, G.M. *see* McISAAC, R.L., 434P
WILSON, K.A. *see* CROCKER, ANN D., 59
WOODRUFF, G.N. *see* ELKHAWAD, A.O., 456P
WOODS, H.F. *see* BOULLIN, D.J., 121
WOODS, H.F. *see* GRAHAM, C.W., 450P

Y

YAMADA, S. *see* HAYASHI, E., 207
YOUDIM, M.B.H. *see* GRAHAM, C.W., 450P
YOUDIM, M.B.H. *see* PARVEZ, SIMONE, 241
YOULTEN, L.J.F. *see* HIGGS, G.A., 539
YOUNG, ANNE B. *see* SNYDER, S.H., 473
YOUNG, J.M. *see* ROBINSON, D.A., 363
YOUNG, S.N. *see* GREEN, A.R., 287

Z

ZIMMERMAN, B.G. *see* KRAFT, E., 51

SUBJECT INDEX

A

AH 5158, combined α- and β-adrenoceptor blocking drug, 585
– , 5-[1-hydroxy-2-[(1-methyl-3-phenylpropyl)-amino]ethyl]salicylamide, 585
AH 10407, a novel short-acting competitive neuromuscular blocking drug in man and animals, 446P
Acebutolol, effect of, on cardiovascular and bronchodilator responses to isoprenaline, 173
Acetylcholine, effect of, on changes in contractility, heart rate and phosphorylase activity produced by isoprenaline, salbutamol and aminophylline in perfused guinea-pig heart, 531
– , effect of, on cloacal temperature in pigeon, 317
– , effect of, on myenteric plexus-longitudinal muscle, 131
– , effect of, on neonatal rabbit ileum, 428
– , effect of surugatoxin on effects of, 207
– , energy dependence of contractions of isolated smooth muscle induced by, 59
– , increases antagonistic effect of 2-2'pyridylisatogen tosylate to ATP, 575
– , induced currents, effect of hyperpolarization on, at frog endplates, 308
– , potentiation and antagonism of excitatory responses to, following application of imipramine or desipramine, 29
– , sensitivity of brain stem cells to, 462P
Acetylcholine mustard, irreversible binding of, to muscarinic receptors in intestinal smooth muscle of the guinea-pig, 363
***N*-acetylcysteine**, effect of, on toxicity of M & B 16,573 in cat, 93
Acetyl-β-methylcholine, sensitivity of brain stem cells to, 462P
ACTH, activity of, as measure of effect of reserpine on hypothalamo-pituitary-adrenocortical function in the rat, 555
Adenosine 5'-triphosphate, antagonism of relaxation induced by, in taenia of guinea-pig caecum, by 2-2'pyridylisatogen, 575
Adrenal, stimulation of, by compounds formed in inflamed tissue, 75
Adrenal medulla, rat, effect of reserpine on, in spontaneous hypertension, 349
Adrenaline, contractile response of vascular tissue to, role of extraneuronal mechanisms in termination of, 267
– , effect of, on histamine constricted guinea-pig lung, 463P
– , effect of surugatoxin on effects of, 207
– , effects of dexamethasone on release of, in adult and neonatal rat brain, 453P
– , effects of, on canine peripheral canine lymph flow, 499
– , effects of, on production and release of renin by isolated renal cortical cells of cat, 67
– , effect of, on ventricular fibrillation threshold blocked by propranolol and pindolol, 3
– , lowers ventricular fibrillation threshold in isolated rabbit heart, 3
– , lowering effect of, on ventricular fibrillation threshold in isolated rabbit heart unaffected by carbachol, 3
– , uptake, effect of reserpine on, in adrenal medulla of spontaneously hypertensive rat, 349
(±)-Adrenaline, uptake of, by membranes of adrenal chromaffin granules, 257
$[^3H]$-adrenaline, metabolism of, in rat liver during pregnancy, 241
Adrenergic nerves, responsibility of, for uptake of noradrenaline in guinea-pig isolated trachea, 109
Adrenergic neurone-blocking action, of propranolol, 517
α-adrenoceptor-blocking drugs, effect of, on vascular response to sympathetic nerve stimulation, 517
α-adrenoceptor, action of antagonists on response to isoprenaline in oestrogen dominated rabbit uterus, 393
– , agonists and antagonists effects of, on release of ^{3}H-(–)-noradrenaline from guinea-pig hypothalamic slices, 454P
– , effect of aminophylline on, blockade, 163
– , further studies on blocking of, in anaesthetized animals, 585
β-Adrenoceptor, action of, antagonists on response to isoprenaline in the oestrogen dominated rabbit uterus, 393
– , effect of aminophylline on, blockade, 163
– , mediate effect of adrenaline, noradrenaline and isoprenaline on ventricular fibrillation threshold in isolated rabbit heart, 3
– , possible role of, in regulation of noradrenaline release by nerve stimulation through a positive feed-back mechanism, 43
– , role in muscle glycogenolysis in the dog, 157
β_1-adrenoceptors, mediate lipolysis in the dog, 157
β_2-adrenoceptors, mediate liver glycogenolysis in the dog, 157
Adrenoceptor-blocking drugs, effects of, on canine peripheral lymph flow, 499
Adrenocorticotrophic hormone, effect of, on nicotine-induced steroidogenesis in adrenocortical cells of rat, 357
Agonist conductance changes, kinetics of, during hyperpolarization at frog endplates, 308
Alcohol, voluntary consumption of, in rat, failure of *p*-chlorophenylalanine to affect, 299

***N*-amidine-2-(2,6,-dichlorophenol) acetamide hydrochloride**, *see* BS 100-141, 513

Amines, contractile responses to, in vascular tissue, role of extraneuronal mechanisms in termination of, 267

γ-aminobutyric acid, actions of, on ganglionic transmission and ganglion cell excitability, 438P

– , effect on membrane conductance at crab neuromuscular junction induced by, and effect of tetramethylene-disulphotetramine, 598

– , depolarization of isolated superior cervical ganglion induced by, inhibited by tetramethylenedisulphotetramine, 422

2-Amino-6,7-dihydroxyl-1,2,3,4-tetrahydronapthalene, effect of, on motor activity in rat, 456P

Aminooxyacetic acid, effect of, on metoclopramide-induced catalepsy in rat striatum, 460P

Aminophylline, action of, on transplanted dog heart, 163

– , changes in contractility, heart rate and phosphorylase activity produced by, in perfused guinea-pig heart, effect of acetylcholine on, 531

– , effect on coronary blood flow and myocardial contractility, 163

– , effect of, on histamine constricted guinea-pig lung, 463P

– , effect of, on membrane potential of vascular endothelial cells, 113

Aminopyrine, effect of, on membrane potential of vascular endothelial cells, 113

Amodiaquin, effect of, on contractile response of vascular tissue to amines, 267

AMP, effect of, on nicotine-induced steroidogenesis in adrenocortical cells in cat, 357

(+)-Amphetamine, comparison of effects of, on rat blood platelets, with (+)-fenfluramine, 563

(+)-Amphetamine, effect of, on avoidance behaviour in selected rat strains, 461P

Angiotensin, energy dependence of contractions of isolated smooth muscle induced by, 59

– , reduction in response to, following exposure to 2,4-dinitrophenol, 59

– , response to, can be aerobic or anaerobic depending on tissue site, 59

Antihistamines, effects of, on sympathetic nerve responses in canine paw, 51

Antihypertensive action, of propranolol, 517

Anti-inflammatory drugs, effect of, on membrane potential of vascular endothelial cells, 113

Anti-inflammatory substances, endogenous, possible occurrence of, in blood of injured rats, 85

Aortic strips, relaxation of, after contraction in response to amines, 267

Apomorphine, effect of, on metoclopramide-induced catalepsy in rat striatum, 460P

– , effects of, on audiogenic seizures in DBA/2 mice, 419

Artery, human umbilical, kinetics and characteristics of noradrenaline uptake by, 152

L-ascorbic acid, inhibitory effect of, on yield of prostaglandin F from guinea-pig uterine homogenates, 469P

Aspirin, effect of, on prostaglandin synthesis in microsomal fraction of rat skin, 470P

ATP, effect of, on uptake of monoamines by membranes of adrenal chromaffin granules, 257

Atria, kitten isolated, indirect sympathomimetic effect of burimamide on, 525

Atropine, effect of, on cardiovascular effects of surugatoxin, 207

– , effect of, on cloacal temperature in pigeon, 317

– , effect of, on gastric secretion and mucosal blood flow in conscious dog, 432P

– , effect of, on metoclopramide-induced catalepsy in rat striatum, 460P

– , effect of, on pentagastrin-induced gastric acid secretion and mucosal blood flow in conscious rat, 434P

– , effect of, on responses to acetylcholine in cortical neurones, 29

– , thermoregulatory changes induced by, in sheep cerebral ventricles, 489

– , vasodilator responses to histamine, bradykinin and sympathetic nerve stimulation in canine paw infused with, 51

B

BS 100-141, inhibitory effects of, on response to sympathetic nerve stimulation in cats and rabbits, 513

Babylonia japonica, Japanese ivory mollusc, pharmacological studies on toxic principle from, 207

Bacterial pyrogen, effect of various amine-depleting drugs on fever response in rabbits to, 21

Benzindamine, effect of, on membrane potential of vascular endothelial cells, 113

Blood, presence of anti-inflammatory substances in, in injured rat, 85

Blood flow, gastric mucosal, effect of burimamide and metiamide on, in cat, 293

– , mucosal, effect of atropine on, in conscious dog, 432P

– , mucosal, effect of atropine on pentagastrin-induced, in conscious rat, 434P

Blood platelets, human, aggregation of, inhibition of, by chlorpromazine, 121

Blood vessels, of cremaster muscle of rat, effect of oxytocin on, 149

Bradykinin, effect of active inflammatory perfusates on response to, in isolated preparations, 75

– , effect of, on myenteric plexus-longitudinal muscle, 131

Brain, isolated perfused rat, 5-HT synthesis in, 450P

Bretylium, uptake of, effect of cocaine and sodium on, in reserpine-treated guinea-pig left atrium, 247

– , vasodilator responses to histamine, bradykinin and sympathetic nerve stimulation in canine paw infused with, 51

British Pharmacological Society, Proceedings of, January 8-10, 1975, 431P

Burimamide, activation of histidine decarboxylase by, in rat stomach, mechanisms of action of, 127

– , effect of, on basal gastric function in cat, 293

– , effect of, on histamine uptake and metabolism in the guinea-pig atrium and mouse neoplastic mast cells, 569

– , effect of, on vasoconstrictor response to exogenous noradrenaline in canine paw, 51

– , effect of, on vasodilator responses in canine paw, 51

– , sympathomimetic effect of, on kitten isolated atria, 525

Butaclamol, effect of, on dopamine receptors in rat striatum, 457P

Betamethasone, effect of, on prostaglandin synthesis in microsomal fraction of rat skin, 470P

Butoxamine, effect of, on cardiovascular and bronchodilator responses to isoprenaline, 173

***n*-Butyl alcohol**, metabolism of, in rat isolated liver, 443P

6-*n*-butyl-2,8-dicarboxy-4,10-dioxo-1,4,7,10-tetrahydro-1,7-phenanthroline, *see* ICI 74,917, 409

C

Ca^{++}, metabolism of, in smooth and skeletal muscle, in response to TMB-8, 279

Calcium, effect of on depolarization produced by anti-inflammatory drugs in vascular endothelial cells, 113

– , effect of, on nicotine-induced steroidogenesis in adrenocortical cells of cat, 357

Carbachol, contractile response of, abolished byacetylcholine mustard at high concentrations, 363

– , effect of desipramine on responses to, in cortical neurones, 29

– , effect of, on cloacal temperature in pigeon, 317

– , increases antagonistic effect of 2-2′pyridylisatogen tosylate to ATP, 575

– , induced currents, effect of hyperpolarization on, at frog endplates, 308

– , lowering effect of adrenaline on ventricular fibrillation threshold in isolated rabbit heart unaffected by, 3

– , thermoregulatory changes induced by, in sheep cerebral ventricle, 489

Cardiac sympathetic nerves, stimulant effects of 5-HT on, 449P

Carrageenin, inflammatory effects of active perfusates of, on responses of isolated preparations to histamine, bradykinin, prostaglandins and 5-HT, 75

Catechol-*O*-methyltransferase, activity of, in rat liver, during pregnancy, 241

– , effect of inhibition of, on contractile response of vascular tissue to amines, 267

Catecholamines, effects of, on canine peripheral lymph flow, 499

– , in adrenal medulla of spontaneously hypertensive rat, effect of reserpine on, 349

– , in isolated cat cerebral cortex, 455P

– , release of, from kitten isolated atria, by burimamide, 525

Cells, adrenocortical, cat, stimulation of nicotine-induced steroidogenesis in, 357

– , vascular endothelial, membrane potential of, effect of anti-inflammatory drugs on, 113

Cerebral ventricles, sheep, thermoregulatory changes induced by cholinomimetic substances introduced into, 489

***N*-2-chloroethyl-*N*-methyl-2-acetoxyethylamine**, *see* acetylcholine mustard, 363

Chloroform, lowering effect of adrenaline on ventricular fibrillation threshold in isolated rabbit heart potentiated by, 3

– , lowering effect of isoprenaline on ventricular fibrillation threshold in isolated rabbit heart unaffected by, 3

Chloroimipramine, comparison of effects of, on rat blood platelets, with (+)-fenfluramine, 563

***p*-Chlorophenylalanine**, failure of, to affect voluntary alcohol consumption in rat, 299

– , pretreatment with, in combination with α-methyltyrosine slightly enhances fevers produced by bacterial and leucocyte pyrogen, 21

– , pretreatment with, slightly enhances fever response to both bacterial and leucocyte pyrogen in rabbit, 21

4-(*m*-Chlorophenylcarbamoyloxy)-2-butynyl-trimethylammonium chloride, effect of, affected by surugatoxin in anaesthetized cats, 207

Chloroquine, effect of, on membrane potential of vascular endothelial cells, 113

Chlorpheniramine, effect of, on histamine uptake and metabolism in guinea-pig atrium and mouse neoplastic mast cells, 569

Chlorpromazine, and metabolites, distribution of, in subfractions of rat brain, 451P

– , and metabolites, inhibition of 5-hydroxytryptamine-induced human blood platelet aggregation by, 121

– , effect of, on β-adrenoceptor system in rat striatum, 457P

Chlorpromazine sulphoxide, inhibition of 5-hydroxytryptamine-induced human blood platelet aggregation by, 121

[^{3}H]-choline, uptake and subcellular distribution of, in retina, 447P

Cholinergic mechanisms, in central thermoregulation in pigeon, 317

Cholinomimetic substances, thermoregulatory changes induced by, introduced into cerebral ventricles of sheep, 489

Chorisondamine, effect of, on effect of reserpine on adrenal medulla in spontaneously hypertensive rat, 349

Chromaffin granules, adrenal, membranes of, discrimination of monoamine uptake in, 257

Cimetidine, a new histamine H$_2$-receptor antagonist, 435P

Cinchocaine, effect of, on membrane potential of vascular endothelial cells, 113

Clonidine, inhibitory effects of, on responses to sympathetic nerve stimulation in cats and rabbits, 513

Clozapine, bioanalytical assay of, and its N-oxide metabolite and determination of blood levels in dog, 440P

Cocaine, effect of, on contractile response of vascular tissue to amines, 267

– , effect of, on histamine uptake and metabolism in guinea-pig atrium and mouse neoplastic mast cells, 569

– , effect of, on neuronal uptake of noradrenaline, 517

– , effect of, on response of kitten isolated atria to burimamide, 525

– , effect of, on uptake of noradrenaline by human umbilical artery, 152

– , influence of, on bretylium uptake by reserpine-treated guinea-pig left atrium, 247

– , pretreatment with, effect of AH 5158 on noradrenaline pressor responses, 585

Concavalin A, induced inflammation in rat, mechanisms of, 445P

Contractile responses, termination of, role of extraneuronal mechanisms on, 267

Contractility, changes in, effect of acetylcholine on, produced by isoprenaline, salbutamol and aminophylline in perfused guinea-pig heart, 531

Corticotrophin, adaptation of release of, to administration of reserpine, 555

Cortisol, effect of, on lung inactivation of prostaglandin E_2, 547

Cross-desensitization, in guinea-pig ileum, 448P

Cyclazocine, effect of, on myenteric plexus-longitudinal muscle, 131

Cyclic AMP, effect of dopamine receptor stimulants on levels of, in rat striatum, 456P

Cyclic adenosine 3',5'-monophosphate, role of, in isoprenaline-induced relaxation of oestrogen dominated rabbit uterus, 403

Cycloheximide, effect of, on prostaglandin synthesis, 467P

– , responsiveness to analgesic effects of morphine administered together with, in rats, 383

N-**cyclohexylmethyl-*N*-ethyl-β-chloroethylamine**, *see* GD-131, 267

Cysteamine, effect of, on toxicity of M & B 16,573 in cat, 93

Cysteine, effect of, on effect of M & B 16,573 in cat, 93

D

Depolarization, of membranes of vascular endothelial cells, effect of anti-inflammatory drugs on, 113

Desimipramine, effect of, on neuronal monoamines in cerebral cortex and corpus striatum, 459P

Desipramine, effect of, on excitatory responses to acetylcholine in cortical neurones, 29

– , effect of, on response to carbachol in cortical neurones, 29

Desmethylimipramine, effect of, on neuronal uptake of noradrenaline, 517

Dexamethasone, effect of, on prostaglandin synthesis in microsomal fraction of rat skin, 470P

– , effects of, on phenylethanolamine N-methyltransferase and adrenaline in adult and neonatal rat brain, 453P

Dexamphetamine, induction of drowsiness by, infused into the hypothalamus of fowl, 193

Dexpropranolol, effect of, on vascular responses to sympathetic nerve stimulation, 517

Dextrorphan, effect of, on brain stem neurones, 462P

– , effect of, on myenteric plexus-longitudinal muscle, 131

L-2,4-diaminobutyric acid, as a selective marker for inhibitory nerve terminals in rat brain, 439P

Dibenzoxazepine, effect of, on sensory nerves, 436P

Dibutyryl cyclic adenosine 3',5'-monophosphate, effect of, in rat gastric mucosa, 217

Dibutyryl cyclic AMP, effect of, compared with that of isoprenaline in oestrogen dominated rabbit uterus, 304

Didesmethylchlorpromazine, inhibition of 5-hydroxytryptamine-induced human blood platelet aggregation by, 121

Didesmethylchlorpromazine sulphoxide, inhibition of 5-hydroxytryptamine-induced blood platelet aggregation by, 121

8-(*N*,*N*-diethylamino)octyl 3,4,5-trimethoxy benzoate hydrochloride, a new Ca^{++} antagonist, action of, in smooth and skeletal muscle, 279

Dihydroergotoxine, effect of, on canine peripheral lymph flow, 499

3,4-Dihydroxy-2-methyl propiophenone, *see* U-0521, 267

3,4-Dihydroxyphenylacetic acid, estimation of, in nervous tissue by gas-liquid chromatography and electron capture detection, 143

Dimercaprol, effect of, on toxicity of M & B 16,573 in cat, 93

2,6-Dimethoxybenzoquinone, effect of, in cat, 93

3,7-Dimethoxychlorpromazine, inhibition of 5-hydroxytryptamine-induced human blood platelet aggregation by, 121

2,6-Dimethoxyphenol, and its ester, toxicity of, prevention of, by sulphydril compounds in cat, 93

(–)-2,6-Dimethoxypheyl-2-morpholino propionate hydrochloride, *see* M & B 16,573

2,6-Dimethoxyquinol, effect of, in cat, 93

1,1-Dimethyl-4-phenylpiperazinium iodide, effect of surugatoxin on effects of, 207

2,4-Dinitrophenol, effect of, on angiotensin response of smooth muscle, 59

Di-4-phloretin phosphate, effect of, on pulmonary inactivation in rabbits *in vivo*, 467P

Dipyridamole, action of nitroglycerine and, in normal and ischaemic dog heart, 11

– , depresses heat production in normal and ischaemic myocardium, 11

– , depression of myocardial heat production by, may be result of action on efficiency of oxygen utilization during cardiac contraction, 11

– , dilates network and post-network vessels equally effectively in ischaemic dog heart, 11

– , increases heat clearance in normal but not ischaemic myocardium, 11

DOPAC, *see* 3,4-dihydroxyphenylacetic acid, 143

Dopamine, action of apomorphine, ergocornine and piribedil as agonists of, in audiogenic seizures in DBA/2 mice, 419

– , concentration of, in rabbit brainstem during bacterial or leucocyte pyrogen fever, 21

– , metabolites of, estimation of, in nervous tissue by gas-liquid chromatography and electron capture detection, 143

– , effect of metoclopramide under influence of various drugs, on striatal content of, 460P

– , effect of, on waking and sleeping in fowl, 193

– effect of stimulants on receptors for, on locomotor activity and cyclic AMP levels in rat striatum, 456P

– , quaternary methyl derivative of, thermoregulatory changes induced by, in sheep cerebral ventricles, 489

– , uptake of, by membranes of adrenal chromaffin granules, 257

Dopamine-β-hydroxylase, activity of, in human plasma following intravenous theophylline, 425

– , in adrenal medulla of spontaneously hypertensive rat, effects of reserpine on, 349

[^{3}H]-dopamine, synthesis of, in cat cerebellar cortex, 455P

Dose ratios, calculation of, use of logistic function for, 139

Drug desensitization, in guinea-pig ileum, 448P

E

***E. coli* endotoxin**, protective effect of sodium meclofenamate in experimental, shock, 466P

– , release of prostaglandins and incapacitation following injection of, in dog knee joint, 465P

– , endotoxin shock, in cat, treatment of, with indomethacin, 485

Electrical stimulation, effect of, on noradrenaline release in cat nictitating membrane and guinea-pig ileum myenteric plexus-longitudinal muscle preparation, 505

Electron capture detection, use of to estimate homovanillic acid, homo-isovanillic acid and 3,4-dihydroxyphenylacetic acid in nervous tissue, 143

Endotoxin shock, *E. coli*, in cat, treatment of, with indomethacin, 485

Endplates, frog, agonist conductance changes during hyperpolarization at kinetics of, 308

Ergocornine, effects of, on audiogenic seizures in DBA/2 mice, 419

Ergometrine, effect of, on motor activity in rat, 456P

Ergotamine, effect of, on canine peripheral lymph flow, 499

ET 495, *see* Piribedil, 419

Ethanol, effect of, on hepatic microsomal metabolism of foreign compounds in rat, 442P

Ethyl alcohol, metabolism of, by rat isolated liver, 443P

N-ethylmaleimide, effect of, on monoamine uptake by membranes of adrenal chromaffin granules, 257

n-Ethyl 3-piperidyl benzilate, effect of, on avoidance behaviour in selected rat strains, 461P

Extraneuronal mechanisms, role of, in termination of contractile responses to amines in vascular tissue, 267

F

Fenfluramine, effect of, on 5-hydroxytryptamine uptake and release by rat blood platelets, 563

(+)-Fenfluramine, comparison of effects of, with chloroimipramine, (+)-amphetamine, (−)-fenfluramine and (±)-norfenfluramine, 563

Flufenamic acid, effect of, on membrane potential of vascular endothelial cells, 113

Fluphenazine, effect of, on tissue noradrenaline concentrations and interaction with pargyline, 593

Flupenthixol, effect of, on glucagon-sensitive adenylate cyclase system in rat striatum, 457P

Fluorescence histochemistry, use of, to demonstrate destruction of adrenergic nerve terminals, 109

Fluocinolone acetonide, effect of, on prostaglandin synthesis in microsomal fraction of rat skin, 470P

– , effect of, on prostaglandin synthesis in microsomal fraction of rat skin, 470P

G

GD-131, effect of, on contractile response of vascular tissue to amines, 267

Gaddum Memorial Lecture, Fifth, The glycine synaptic receptor, 473

Ganglion cell excitability, action of GABA on, 438P

Ganglionic transmission, action of GABA on, 438P

Gas-liquid chromatography, use of, to estimate homovanillic, homo-isovanillic and 3,4-dihydroxyphenylacetic acids, 143

Gastric acid secretion, effect of burimamide on, in cat, 293

– , effect of atropine on pentagastrin-induced, in conscious rat, 434P

Gastric secretion, effect of atropine on, in conscious dog, 432P

Gastrin, serum concentration, effect of histamine H_2-receptor antagonists on, 127

Glucose, effects of isoprenaline and salbutamol on levels of, in the dog, 157

Glutamate, action of, on lobster muscle fibre and frog spinal cord, 437P

Glycine synaptic receptor, in mammalian CNS, 473

Glycogen, correlation of tissue levels of, with differences in energy requirements for angiotensin II- and acetylcholine-induced contractions in isolated smooth muscle, 59

Guanethidine, effect of, on motor transmission in mouse vas deferens, 323

– , effects of various vasodilator responses in canine paw infused with, 51

H

H_2-receptor blockade, activation of histidine decarboxylase by, 127

Haloalkylamine, effect of, on contractile response of vascular tissue to amines, 267

Halothane, change in sensitivity to, induced by administration of central nervous system depressant drugs, 452P

Heart, guinea-pig perfused, changes in contractility, heart rate and phosphorylase activity in, produced by isoprenaline, salbutamol and aminophylline, effect of acetylcholine on, 531

Heart rate, changes in, effect of acetylcholine on, produced by isoprenaline, salbutamol and aminophylline in perfused guinea-pig heart, 531

Hepatic microsomal metabolism, effect of ethanol on, in rat, 442P

Hexamethonium, effect of, on myenteric plexus-longitudinal muscle, 131

Histamine, effect of active inflammatory perfusates on response to, in isolated preparations, 75

– , contractile response of vascular tissue to, role of extraneuronal mechanisms in, 267

– , effect of, on membrane potential of vascular endothelial cells, 113

– , dose response relationship and comparison of secretory potency of methyl histamine on isolated guinea-pig stomach, 432P

– , effect of, on myenteric plexus-longitudinal muscle, 131

– , effect of surugatoxin on effects of, 207

– , effects of, on isolated cat nictitating membrane, 431P

– , influence of, H_1 and H_2-receptor blockers on sympathetic vasodilator and vasoconstrictor responses in canine paw, 51
– , interaction of H_1 and H_2-receptor antagonists with histamine uptake and metabolism by guinea-pig isolated atrium and mouse neoplastic mast-cells *in vitro*, 569
– , sensitivity of neonatal rabbit ileum to, 428
– , uptake of, by membranes of adrenal chromaffin granules, 257
Histidine decarboxylase, activation of, by H_2-receptor blockade, 127
Homo-isovanillic acid, estimation of, in nervous tissue by gas-liquid chromatography and electron capture detection, 143
– , effect of metoclopramide on striatal content of, under influence of various drugs, 460P
– , estimation of, in nervous tissue by gas-liquid chromatography and electron capture detection, 143
HVA, *see* homovanillic acid, 143
iso-**HVA,** *see* homo-isovanillic acid, 143
Hydrocortisone, effect of, on prostaglandin synthesis in microsomal fraction of rat skin, 470P
– , effect of, on membrane potential of vascular endothelial cells, 113
– , effect on spontaneous mechanical activity of veins, 183
– , treatment with, effect of, on liver and brain metabolism or tryptophan in rat and gerbil, 287
7-Hydroxychlorpromazine, inhibition of 5-hydroxytryptamine-induced human blood platelet aggregation by, 121
6-Hydroxydopamine, destruction of adrenergic nerve terminals by, in guinea-pig isolated trachea, 109
– , effect of, on renal hypertension in rabbit, 455P
3-Hydroxy-4-methoxyphenylacetic acid, *see* homo-isovanillic acid, 143
4-Hydroxy-3-methoxyphenylacetic acid, *see* homovanillic acid, 143
5-[1-Hydroxy-2-[(1-methyl-3-phenylpropyl)amino]-ethyl]salicylamide, *see* AH 5158, 585
5-Hydroxytryptamine, central effects of, in young chickens at and below thermoneutrality, 37
– , concentration of, in rabbit brainstem during bacterial or leucocyte pyrogen fever, 21
– , contractile response of vascular tissue to, role of extraneuronal mechanisms in, 267
– , effect of active inflammatory perfusates on response to, in isolated preparations, 75
– , effect of, on waking and sleeping in fowl, 193
– , -induced human blood platelet aggregation, inhibition of, by chlorpromazine and its metabolites, 121
– , possible role of, as thermoregulatory transmitter in rabbit, 21
– , salts of, produce behavioural sleep in young chickens below thermoneutrality, 37
– , stimulant effect of, on cardiac sympathetic nerves, 449P
– , synthesis in isolated perfused rat brain, 450P
– , uptake and release of, effect of fenfluramine on, in rat blood platelets, 563
– , uptake of, by membranes of adrenal chromaffin granules, 257
5-Hydroxytryptamine creatinine sulphate, produces hypothermia in small doses and hyperthermia at higher doses in young chickens, 37
5-Hydroxytryptamine hydrogen maleinate, produces hypothermia in young chickens, 37
Hyoscine, effect of, on myenteric plexus-longitudinal muscle, 135
Hyperpolarization, at frog endplates, agonist conductance changes during, kinetics of, 308
Hypertension, spontaneous, in rat, effect of reserpine on, 349
Hyperthermia, effect of cholinergic mechanisms on, in pigeon, 317
– , effect of cholinergic mechanisms on, in pigeon, 317
Hypothalamus, stimulation of, by compounds formed in inflamed tissue, 75

I

Ibuprofen, effect of, on prostaglandin synthesis in microsomal fraction of rat skin, 470P
ICI 74,917, effects of, in certain laboratory animals, 409
– , inhibition of immediate hypersensitivity reactions in laboratory animals by, 409
– , novel anti-allergic compound, 409
Imipramine, effect of, on excitatory responses to acetylcholine in cortical neurones, 29
Indomethacin, effect of, on corpus luteum regression in the guinea-pig, 225
– , effect of, on incapacitation of dog knee joint following injection of endotoxin, 465P
– , effect of, on membrane potential of vascular endothelial cells, 113
– , effect of, on noradrenaline release in rabbit, 596
– , effect of, on prostaglandin synthesis in microsomal fraction of rat skin, 470P
– , effect of, on prostaglandins in rat gastric mucosa, 217
– , effect of, on protective effect of sodium meclofenamate in experimental endotoxin shock, 466P
– , potentiation by, of response of rabbit portal vein to electrical stimulation, 596
– , treatment of *E. coli* endotoxin shock with, 485
Inflammation, stimulation of hypothalamo-pituitary-adrenal axis by compounds formed in inflamed tissue, 75
Inflammatory lesion, production of prostaglandin by, in sheep, 305
Inhibitory nerve terminals, rat brain, L-2,4-diaminobutyric acid as a selective marker for, 439P
Iprindole, effect of, on neuronal monoamines in cerebral cortex and corpus striatum, 459P
Iproniazid, effect of, on contractile response of vascular tissue to amines, 267
Iso-**amyl alcohol,** metabolism of, in rat isolated liver, 443P
Isoprenaline, action of β-adrenoceptor antagonists on the response to, in oestrogen dominated rabbit uterus, 393
– , changes in contractility heart rate and phosphorylase activity produced by, in perfused guinea-pig heart, effect of acetylcholine, 531

– , effect of, on canine peripheral lymph flow, 499
– , effect of, on cyclic AMP in oestrogen dominated rabbit uterus, 403
– , effect of, on histamine constricted guinea-pig lung, 463P
– , effect of, on membrane potential of vascular endothelial cells, 113
– , effect of, on noradrenaline overflow produced by nerve stimulation in guinea-pig isolated atria, 43
– , effect of, on oestrogen dominated rabbit uterus, 403
– , effect of, on ^{86}Rb uptake by horse lymphocytes *in vitro*, 448P
– , effects of, on production and release of renin by isolated renal cortical cells of cat, 67
– , i.c.v., tachycardia produced by in mice, 341
– , lack of cardiac or bronchodilator tachyphylaxis to, in dog, 333
– , lowers ventricular fibrillation threshold in isolated rabbit heart, 3
– , metabolic and cardiovascular effects of, in the dog, 157
– , selectivity of β-adrenoceptor antagonists on cardiovascular and bronchodilator responses to, in anaesthetized dog, 173
– , uptake by horse lymphocytes *in vitro*, effect of ^{86}Rb on, 448P

K

Kainic acid, action of, on lobster muscle fibre and frog spinal cord, 437P
Kallikreins, synthesis of, by rat kidney slices, 229
Ketoprofen, effect of, on corpus luteum regression in the guinea-pig, 225

L

Lactic acid, effects of isoprenaline and salbutamol on levels of, in the dog, 157
Lanthanum, effect of, on membrane potential of vascular endothelial cells, 113
Lesion, inflammatory, in rats, production of anti-inflammatory substances by, 85
– , mild inflammatory, production of prostaglandin by, in sheep, 305
$[^{3}H]$-L-leucine, isolation of radioactive kallikreins from rat kidney slices incubated with, 229
Leucocyte pyrogen, effect of various amine-depleting drugs on fever response in rabbits to, 21
Levorphanol, effect of, on brain stem neurones, 462P
– , effect of, on myenteric plexus-longitudinal muscle, 131
Lithium, distribution of, in rat brain and plasma following administration of lithium in diet, 235
Liver, rat isolated, metabolism of alcohols by, 443P
Logistic function, use of, for calculation of dose-ratios and potency ratios, 139
Lymph flow, peripheral canine, effects of catecholamines and adrenoceptor-blocking drugs on, 499

M

M & B 16,573, toxicity of, in cat, prevention of, by sulphydril compounds, 93
Magnesium, distribution of, in rat brain and plasma following administration of lithium in diet, 235
Mebanazine, effect of, on waking and sleeping in fowl, 193
Meclofenamic acid, effect of, on membrane potential of vascular endothelial cells, 113
– , effect of, on prostaglandin synthesis in microsomal fraction of rat skin, 470P
Meclofenamate, effect of, on prostaglandins in rat gastric mucosa, 217
Membrane potential, of vascular endothelial cells, effect of anti-inflammatory drugs, on, 113
Mepyramine, variation in number of receptors sensitive to, related to age in rabbit ileum, 428
$[^{3}H]$-metanephrine, metabolism of, in rat liver during pregnancy, 241
(±)-Metaraminol, uptake of, by membranes of adrenal chromaffin granules, 257
α-Methyl dichloroisoprenaline, effect of, on cardiovascular and bronchodilator responses to isoprenaline, 173
Methyl histamine, dose response relationship and comparison of secretory potency of, with histamine on isolated guinea-pig stomach, 432P
***N*-methyl-*N*′-4(-[4(5)-imidazolyl] butyl thiourea)**, burimamide, 51
Methyltryptamine, effects of, on waking and sleeping in fowl, 193
α-Methyltyrosine, pretreatment with, in combination with *p*-chlorophenylalanine slightly enhances fevers produced by bacterial and leucocyte pyrogen, 21
– , pretreatment with, reduces fever response to both bacterial and leucocyte pyrogen in rabbit, 21
Methysergide, prevents hypothermia produced by 5-hydroxytryptamine hydrogen maleinate, 37
Metiamide, activation of histidine decarboxylase by, in rat stomach, mechanism of action of, 127
– , effect of, on basal gastric function in cat, 293
– , effect of, on histamine uptake and metabolism in the guinea-pig atrium and mouse neoplastic mast cells, 569
– , effect of, on kitten isolated atria, 525
– , effects of, on vasodilator responses in canine paw, 51
Metoclopramide, cataleptic action of, affected by drug induced alteration of dopaminergic or cholinergic activity or GABA content of rat striatum, 460P
Monoamine-metabolizing enzymes, variation in activity of, in rat liver during pregnancy, 241
– , oxidase, activity of, in rat liver during pregnancy, 241
Monoamine oxidase, effect of inhibition of, on contractile response of vascular tissue to amines, 267
– , effects of age on irreversible inhibition of cardiac, 458P
– , inhibition of, intensifies magnitude and response of 5-hydroxytryptamine hydrogen maleinate at thermoneutrality, 37
Monoamines, relationship between uptake blockade and potentiation of neuronal responses, effects of, 459P

Monoamine uptake, discrimination of, by membranes of adrenal chromaffin granules, 257
Monodesmethylchlorpromazine, inhibition of 5-hydroxytryptamine-induced human blood platelet aggregation by, 121
Morphine, acute tolerance to, in guinea-pig isolated ileum, an analysis of. 131
– , effect of, on adrenergic transmission in mouse vas deferens, 371
– , effect of, on release of noradrenaline from cat isolated nictitating membrane and guinea-pig ileum myenteric plexus-longitudinal muscle preparation, 505
– , microiontophoretic study of stereospecific action of, on brain stem neuronal activity, 462P
– , offset of tolerance to, in rats and mice, 383
Morphine receptors, of myenteric plexus-longitudinal muscle, involvement of, in acute tolerance to morphine, 131
Morpholinopropionyl ester, of 2,6-dimethoxyphenol, prevention of toxicity of, by sulphydryl compounds in cat, 93
Muscle, cremaster, of rat, effect of oxytocin on blood vessels of, 149
– , isolated papillary, guinea-pig, kinetics of [^{3}H]-ouabain uptake in, 99
– , smooth and skeletal, action of a new Ca^{++} antagonist, TMB-8, in, 279
– , myenteric plexus-longitudinal, morphine receptors of, 131
Myenteric plexus-longitudinal muscle preparation, guinea-pig ileum, effect of morphine on noradrenaline release in, 505

N

NADPH, dependent warfarin reductase in human and rat liver and kidney and soluble fraction, 441P
Nalorphine, effect of, on myenteric plexus-longitudinal muscle, 131
Naloxone, antagonizes effect of morphine on mouse vas deferens, 371
– , antagonism of morphine-induced depression of brain stem cell firing by, 462P
– , effect of, on noradrenaline release from cat nictitating membrane and guinea-pig ileum myenteric plexus-longitudinal muscle preparation, 505
– , effect of, on myenteric plexus-longitudinal muscle, 131
Naltrexone, antagonizes effect of morphine on mouse vas deferens, 371
Naproxen, effect of, on prostaglandin synthesis in microsomal fraction of rat skin, 470P
Narcotic analgesics, comparison of effects of various, on noradrenaline output and longitudinal contraction of mouse vas deferens, 371
Nerve, sympathetic, stimulation of, inhibitory effects of clonidine and BS 100-141, 513
– , sympathetic, stimulation of, vascular response to, effect of propranolol on, 517
Nerve stimulation, responses of rat heart to, effect of vinblastine or vincristine on, 444P
Neuronal uptake, effect of inhibition of, on contractile response of vascular tissue to amines, 267
Nialamide, effect of, on monoamine-metabolizing enzymes in rat liver during pregnancy, 241
Nicergoline, effect of, on canine peripheral lymph flow, 499
Nicotine, effect of, on adrenal medulla of spontaneously hypertensive rat, 349
– , thermoregulatory changes induced by, in sheep cerebral ventricles, 489
– , stimulation of steroidogenesis in adrenocortical cells of cat, induced by, 357
Nictitating membrane, cat, effects of morphine on noradrenaline release in, 505
– , isolated cat, effects of histamine on, 431P
– , responses of, induced by stimulation of sympathetic nerve effect of clonidine on, 513
Nitroglycerine, action of, of dipyridamole in normal and ischaemic dog heart, 11
Nitroglycerine, dilates network vessels twice as effectively as post-network vessels in ischaemic dog heart, 11
– , dilates resistance vessels in ischaemic dog heart, 11
– , effectiveness of, not entirely dependent on coronary vasodilator properties, 11
– , reduces both diastolic and systolic blood pressures in dog, 11
N-nonoyl-vanillylamide, effect of, on sensory nerves, 436P
Noradrenaline, and motor transmission in mouse vas deferens, 323
– , concentration of, in rabbit brainstem during bacterial or leucocyte pyrogen fever, 21
– , contractile response of vascular tissue to, role of extraneuronal mechanisms in transmission termination of, 267
– , effect of burimamide on vasoconstrictor response to, in canine paw, 51
– , effect of fluphenazine on tissue concentrations of, 593
– , effect of, on waking and sleeping in fowl, 193
– , effects of, on peripheral canine lymph flow, 499
– , effects of, on production and release of renin by isolated renal cortical cells of cat, 67
– , lowers ventricular fibrillation threshold in isolated rabbit heart, 3
– , possible role of, as thermoregulatory transmitter in rabbit, 21
– , possible role of a β-adrenoceptor in regulation of release of, by nerve stimulation through a positive feed-back mechanism, 43
– , release of, effect of, morphine on, in cat nictitating membrane and guinea-pig ileum myenteric plexus longitudinal muscle preparation, 505
– , uptake of, kinetics and some characteristics of, by human umbilical artery, 152
– , uptake of, in guinea-pig isolated trachea, responsibility of adrenergic nerves for, 109
– , vasopressor responses induced by, effect of AH 5158 on, 585
^{3}H-(–)-noradrenaline, effects of adrenoceptor agonists and antagonists on release of, from guinea-pig hypothalamic slices, 454P
[^{3}H]-noradrenaline, synthesis of, in cat cerebellar cortex, 455P
(–)-Noradrenaline, uptake of, by membranes of adrenal chromaffin granules, 257

Norfenfluramine, comparison of effects of, on rat blood platelets, with (+)-fenfluramine, 563
Normetanephrine, contractile response of vascular tissue to, role of extraneuronal mechanisms in, 267
– , effect of, on uptake of noradrenaline by human umbilical artery, 152
Normorphine, comparison of effects of, with morphine on mouse vas deferens, 371
– , effect of, on cat nictitating membrane and guinea-pig ileum myenteric plexus-longitudinal muscle preparation, 505

O

(+)-Octopamine, uptake of, by membranes of adrenal chromaffin granules, 257
Oedema, carrageenin rat paw, use of, in determination of anti-inflammatory substances in injured rat, 85
Oestradiol, effects of, following β-adrenoceptor blockade, 183
Oestradiol-17-β, inhibitory action of, on venous smooth muscle, 183
Oestradiol monobenzoate, effect of, on lung inactivation of prostaglandin E_2, 547
17β-Oestradiol, effect of, on contractile response of vascular tissue to amines, 267
Oestrogen, action of β-adrenoceptor antagonists on response to isoprenaline in rabbit uterus dominated by, 393
Ouabain, effect of, on monoamine uptake by membranes of adrenal chromaffin granules, 257
[^{3}H]-ouabain, kinetics of uptake of, and exchange in isolated papillary muscle of guinea-pig, 99
Oxotremorine, thermoregulatory changes induced by, in sheep cerebral ventricles, 489
Oxytocin, effect of, on blood vessels of cremaster muscle of rat, 149

P

Paracetamol, effect of, on prostaglandin synthesis in microsomal fraction of rat skin, 470P
Pargyline, interaction of, with fluphenazine and tissue noradrenaline concentrations, 593
Pempidine, effect of, on tachycardia produced by isoprenaline in mice, 341
– , thermoregulatory changes induced by, in sheep cerebral ventricles, 489
Pentagastrin, effect of metiamide and burimamide on gastric acid secretion stimulated by, in cat, 293
– , gastric acid secretion and mucosal blood flow induced by, effect of atropine on, in conscious rat, 434P
Pentobarbitone, change in sensitivity to, induced by administration of central nervous system depressant drugs, 452P
Phenothiazines, inhibition of 5-hydroxytryptamine-induced human blood platelet aggregation by, 121
Phenoxybenzamine, effect of, on canine peripheral lymph flow, 499
– , effect of, on dose-response curve of isoprenaline in oestrogen dominated rabbit uterus, 393
– , effect of, on noradrenaline release in cat nictitating membrane and guinea-pig ileum myenteric plexus-longitudinal muscle preparation, 505
– , effect of, on response to aminophylline, 163
Phentolamine, effect of, on dose-response curve of isoprenaline in oestrogen-dominated rabbit uterus, 393
– , effect of, on heart rate response to clonidine and BS 100-141, 513
– , effect of, on motor transmission in mouse vas deferens, 323
– , effect of, on noradrenaline overflow produced by nerve stimulation in guinea-pig isolated atria, 43
– , lowering effect of adrenaline on ventricular fibrillation threshold in isolated rabbit heart not influenced by, 3
Phenylbutazone, effect of, on membrane potential of vascular endothelial cells, 113
– , effect of, on prostaglandins in rat gastric mucosa, 217
Phenylephrine, vasopressor responses induced by, antagonized by AH 5158, 585
Phenylethanolamine N-methyltransferase, effects of dexamethasone on release of, in adult and neonatal rat brain, 453P
β-Phenylethylamine, uptake of, by membranes of adrenal chromaffin granules, 257
Phosphorylase activity, changes in, effect of acetylcholine on, produced by isoprenaline, salbutamol and aminophylline in perfused guinea-pig heart, 531
Physostygmine, effect of, on myenteric plexus-longitudinal muscle, 131
– , thermoregulatory changes induced by, in sheep, cerebral ventricles, 489
– , salicylate–effect of, on avoidance behaviour in selected rat strains, 461P
Pindolol, blocks effect of, adrenaline on ventricular fibrillation threshold, 3
Piribedil, effects of, on audiogenic seizures in DBA/2 mice, 419
Pituitary, stimulation of, by compounds formed in inflamed tissue, 75
Potency ratios, calculation of, use of logistic function for, 139
Practolol, effect of, on cardiovascular and bronchodilator responses to isoprenaline, 173
– , effect of, on dose-response curve of isoprenaline in oestrogen dominated rabbit uterus, 393
Pregnancy, activity of monoamine-metabolizing enzymes in rat liver during, 241
Prenylamine, effect of, on waking and sleeping in fowl, 193
Progesterone, effects of, following β-adrenoceptor blockade, 183
– , enhanced inactivation of prostaglandin E_2 in rabbit lung during treatment with, 547
– , inhibitory action of, on venous smooth muscle, 183
Propranolol, blocks effect of adrenaline on ventricular fibrillation threshold, 3
– , effect of, on β-adrenoceptors in transplanted dog heart, 163
– , effect of, on canine peripheral lymph flow, 499
– , effect of, on cardiovascular and bronchodilator responses to isoprenaline, 173

– , effect of, on cardiovascular effects of surugatoxin, 207

– , effect of, on dose-response curve of isoprenaline in oestrogen dominated rabbit uterus, 393

– , effect of, on noradrenaline overflow produced by nerve stimulation in guinea-pig isolated atria, 43

– , effect of, on production of cyclic AMP in oestrogen dominated rabbit uterus, 403

– , effect of, on vascular response to sympathetic nerve stimulation, 517

(+)-Propranolol, effect of, on dose-response curve of isoprenaline in oestrogen dominated rabbit uterus, 393

(–)-Propranolol, effect of, on response of kitten isolated atria to burimamide, 525

n-Propyl alcohol, metabolism of, in rat isolated liver, 443P

[^{3}H]-propylbenzilylcholine mustard, effect of, inhibited by acetylcholine mustard, 363

Prostaglandin, production of, by mild inflammatory lesion in sheep, 305

– , synthesis of, affected by various non-steroid and steroid anti-inflammatory drugs in microsomal fraction of rat skin, 470P

– , synthesis of, inhibited by indomethacin in guinea-pig luteal regression, 225

– , actions of, on arterial system of sheep, 464P

– , chemotactic role for, released from polymorphonuclear leucocytes during phagocytosis, 539

– , effect of active inflammatory perfusates on response to, in isolated preparations, 75

– , inhibition of pulmonary inactivation of, in rabbit *in vivo*, 467P

– , release of, and incapacitation following injection of endotoxin in dog knee joint, 465P

– , vasodilator and antisecretory role of, in rat gastric mucosa, 217

Prostaglandin E$_1$, chemotactic role of, in rabbit polymorphonuclear leucocytes, 539

Prostaglandin E$_2$, chemotactic role of, in rabbit polymorphonuclear leucocytes, 539

– , effect of, on nicotine-induced steroidogenesis in adrenocortical cells in cat, 357

– , enhanced inactivation of, by rabbit lung during pregnancy or progesterone treatment, 547

Prostaglandin F, inhibitory effect of L-ascorbic acid on yield of, from guinea-pig uterine homogenates, 469P

Prostaglandin F$_{2\alpha}$, chemotactic role of, in rabbit polymorphonuclear leucocytes, 539

Prostaglandin synthetase, factors influencing turnover of, 467P

2-2'Pyridylisatogen tosylate, effect of, on ATP-induced relaxation in isolated guinea-pig taenia caeci, 575

Pyrogen, effect of various amine-depleting drugs on fever response in rabbits to bacterial, or leucocyte, 21

R

Receptor theory, an ambiguity in, 311

Renin, production and release of, in isolated renal cortical cells of cat in presence of adrenaline, noradrenaline, isoprenaline or salbutamol, 67

Reserpine, diminishes fever response to bacterial and leucocyte pyrogen in rabbit, 21

– , effects of, on adrenal medulla of spontaneously hypertensive rat, 349

– , effect of, on monoamine-metabolizing enzymes in rat liver during pregnancy, 241

– , effect of, on monoamine uptake by membranes of adrenal chromaffin granules, 257

– , effect of, on hypothalamo-pituitary-adrenocortical function in the rat, 555

– , effect of, on secretion of ACTH, 555

– , effect of, on tachycardia produced by isoprenaline in mice, 341

– , effect of, on waking and sleeping in fowl, 193

– , pretreatment with, effect on response of kitten isolated atria to burimamide, 525

– , -treated guinea-pig left atrium, effect of cocaine and sodium on bretylium uptake in, 247

S

Salbutamol, changes in contractility, heart rate and phosphorylase activity produced by, in perfused guinea-pig heart, effect of acetylcholine on, 531

– , effects of, on production and release of renin by isolated renal cortical cells of cat, 67

– , metabolic and cardiovascular effects of, in the dog, 157

Salicylic acid, effect of, on membrane potential of vascular endothelial cells, 113

Saline, effect of intake of, on effect of 6-hydroxydopamine on renal hypertension in rabbit, 455P

Sodium, effect of, on bretylium uptake in reserpine-treated guinea-pig left atrium, 247

– , distribution of, in rat brain and plasma following administration of lithium in diet, 235

Sodium deficiency, effect of, on uptake of noradrenaline by human umbilical artery, 152

Sodium meclofenamate, protective effect of, in experimental endotoxin shock, 466P

Steroidogenesis, stimulation of, nicotine-induced, in adrenocortical cells of cat, 357

Sulphydryl compounds, prevention of, toxicity of 2,6-dimethoxyphenol and its ester by, in cat, 93

Surugatoxin, pharmacological studies on, 207

Sympathomimetic effect, indirect, of burimamide on kitten isolated atria, 525

T

Tachycardia, induced by stimulation of sympathetic nerve, effect of clonidine on, 513

– , produced by i.c.v. injections of isoprenaline in mice, 341

Tachyphylaxis, cardiac or bronchodilator, response of, to isoprenaline in dog, 333

Tetramethylenedisulphotetramine, effect of, on γ-aminobutyric acid induced depolarization of the isolated superior cervical ganglion of the rat, 422

– , effect of, on membrane conductance increase produced by γ-aminobutyric acid at crab neuromuscular junction, 598

Theophylline, intravenous administration of, changing human plasma dopamine-β-hydroxylase activity, 425
Thermoregulation, role of cholinergic mechanisms in, in pigeon, 317
Thermoregulatory, changes, induced by cholinomimetic substances introduced into cerebral ventricles of sheep, 489
TMB-8, action of, in smooth and skeletal muscle, 279
Tolerance, acute, to morphine, in guinea-pig isolated ileum, and analysis of, 131
Trachea, guinea-pig isolated, responsibility of adrenergic nerves for uptake of noradrenaline in, 109
Triamcinolone acetonide, effect of, on prostaglandin synthesis in microsomal fraction of rat skin, 470P
Tricyclic antidepressants, effect of, on cholinergic responses of single cortical neurones, 29
– , relationship between uptake blockade and potentiation of neuronal responses, effects of, 459P
Tripelennamine, effect on vasodilator response to histamine in canine paw, 51
– , effect on vasodilator response to nerve stimulation in canine paw, 51
Triprolidine, effect of, on histamine uptake and metabolism in guinea-pig atrium and mouse neoplastic mast cells, 569
Tryptamine, effects of, on waking and sleeping in fowl, 193
– , uptake of, by membranes of adrenal chromaffin granules, 257
Tryptophan, liver and brain metabolism of, following hydrocortisone treatment in rats and gerbils, 287
(+)-Tubocurarine, effect of, on cloacal temperature in pigeon, 317
Tyramine, uptake of, by membranes of adrenal chromaffin granules, 267

U

U-0521, effect of, on contractile response of vascular tissue to amines, 267

V

Vas deferens, mouse, effect of noradrenaline on motor transmission in, 323
Ventricular fibrillation, effect of sympathomimetic amines on, threshold in rabbit isolated heart, 3
Vinblastine, effect of, on responses of rat heart to nerve stimulation and to drugs, 444P
Vincristine, effect of, on rat heart responses to nerve stimulation and to drugs, 444P

W

Warfarin, reductase, NADPH-dependent in human and rat liver and kidney soluble fraction, 441P

British Journal of Pharmacology

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CONTENTS

VOLUME 53 NUMBER 1 JANUARY 1975

page

Systematic Pharmacology

3 MURNAGHAN, M.F. The effect of sympathomimetic amines on the ventricular fibrillation threshold in the rabbit isolated heart. (SP1, AP1, DM1)

11 GRAYSON, J. & SCOTT, C. The action of nitroglycerine and dipyridamole in normal and ischaemic dog heart (SP1)

21 METCALF, G. & THOMPSON, J.W. The effect of various amine-depleting drugs on the fever response exhibited by rabbits to bacterial or leucocyte pyrogen. (SP2, AP1)

29 BEVAN, P., BRADSHAW, C.M. & SZABADI, E. The effect of tricyclic antidepressants on cholinergic responses of single cortical neurones. (SP2, DM2)

37 MARLEY, E. & WHELAN, JENNIFER E. Some central effects of 5-hydroxytryptamine in young chickens at and below thermoneutrality. (SP2, DM2)

Autopharmacology

43 ADLER-GRASCHINSKY, EDDA & LANGER, S.Z. Possible role of a β-adrenoceptor in the regulation of noradrenaline release by nerve stimulation through a positive feed-back mechanism. (AP1, PK3, DM2)

51 KRAFT, E. & ZIMMERMAN, B.G. Influence of histamine H_1- and H_2-receptor blockers on sympathetic vasodilator and vasoconstrictor responses in canine paw. (AP2, SP1)

59 CROCKER, ANN D. & WILSON, K.A. A further investigation into the energy dependence of angiotensin II-induced contractions of isolated smooth muscle preparations. (AP3, DM1)

67 JOHNS, E.J., RICHARDS, H.K. & SINGER, BERTHA. Effects of adrenaline, noradrenaline, isoprenaline and salbutamol on the production and release of renin by isolated renal cortical cells of the cat. (AP3, DM2)

75 LEME, J. GARCIA & SCHAPOVAL, ELFRIDES E.S. Stimulation of the hypothalamo–pituitary–adrenal axis by compounds formed in inflamed tissue. (AP3)

Miscellaneous

85 ATKINSON, D.C. & HICKS, R. The possible occurrence of endogenous anti-inflammatory substances in the blood of injured rats. (M)

Pharmacokinetics

93 LOVELESS, A.H. & MAXWELL, D.R. The prevention by sulphydryl compounds of the toxicity in the cat of 2,6-dimethoxyphenol and its morpholinopropionyl ester. (PK2)

99 LULLMANN, H., PETERS, T. & RAVENS URSULA. Studies on the kinetics of [^{3}H]-ouabain uptake and exchange in the isolated papillary muscle of the guinea-pig. (PK3, PK1)

109 FOSTER, R.W. & O'DONNELL STELLA R. Evidence that adrenergic nerves are responsible for the active uptake of noradrenaline in the guinea-pig isolated trachea. (PK3, AP1)

page

Drug Mechanisms

113 NORTHOVER, B.J. Effect of anti-inflammatory drugs on the membrane potential of vascular endothelial cells *in vitro.* (DM1)
121 BOULLIN, D.J., GRAHAME-SMITH, D.G., GRIMES, R.P.J. & WOODS, H.F. Inhibition of 5-hydroxytryptamine-induced human blood platelet aggregation by chlorpromazine and its metabolites. (DM1)
127 HÅKANSON, R., HEDENBRO, J., LIEDBERG, G., REHFELD, J.F. & STADIL, F. Activation of histidine decarboxylase by H_2-receptor blockade: mechanism of action. (DM2, AP2, AP3)
131 KOSTERLITZ, H.W. & WATERFIELD, ANGELA A. An analysis of the phenomenon of acute tolerance to morphine in the guinea-pig isolated ileum. (DM2, AP1)

Pharmacometrics

139 BARLOW, R.B. Use of the logistic function for the calculation of dose-ratios and potency ratios. (PM)
143 PEARSON, J.D.M. & SHARMAN, D.F. The estimation of 3,4-dihydroxyphenylacetic acid, homovanillic acid and homo-isovanillic acid in nervous tissue by gas–liquid chromatography and electron capture detection. (PM, SP2)

SHORT COMMUNICATIONS

149 ELLIOTT, ANNIE B. The effect of oxytocin on blood vessels of cremaster muscle of the rat. (AP3)
152 GULATI, O.D. & SIVARAMAKRISHNA, N. Kinetics and some characteristics of uptake of noradrenaline by the human umbilical artery. (PK3, AP1)

VOLUME 53 NUMBER 2 FEBRUARY 1975

Systematic Pharmacology

157 KELLY, J.G. & SHANKS, R.G. Metabolic and cardiovascular effects of isoprenaline and salbutamol in the dog (SP1, SP3)
163 PAOLONI, H.J. & WILCKEN, D.E.L. The action of aminophylline on the acutely transplanted dog heart: effect of α- and β-adrenoceptor blockade (SP1, DM2)
173 DALY, M.J., FLOOK, JUDITH J. & LEVY, G.P. The selectivity of β-adrenoceptor antagonists on cardiovascular and bronchodilator responses to isoprenaline in the anaesthetized dog (SP1, DM2)
183 McCALDEN, T.A. The inhibitory action of oestradiol-17-β and progesterone on venous smooth muscle (SP1, AP3)
193 MARLEY, E. & NISTICÒ, G. Tryptamines and some other substances affecting waking and sleep in fowls (SP2)

Autopharmacology

207 HAYASHI, E. & YAMADA, S. Pharmacological studies on surugatoxin, the toxic principle from Japanese ivory mollusc (*Babylonia japonica*) (AP1)
217 MAIN, I.H.M. & WHITTLE, B.J.R. Investigation of the vasodilator and antisecretory role of prostaglandins in the rat gastric mucosa by use of non-steroidal anti-inflammatory drugs (AP2)
225 DONOVAN, B.T. Indomethacin, ketoprofen and corpus luteum regression in the guinea-pig (AP2, DM2)
229 NUSTAD, K., PIERCE, J.V. & VAAJE, KIRSTEN. Synthesis of kallikreins by rat kidney slices (AP2, PK2)

page

Pharmacokinetics

235 BOND, P.A., BROOKS, B.A. & JUDD, A. The distribution of lithium, sodium and magnesium in rat brain and plasma after various periods of administration of lithium in the diet (PK1, SP2)

241 PARVEZ, SIMONE, PARVEZ, S.H. & YOUDIM, M.B.H. Variation in activity of monoamine metabolizing enzymes in rat liver during pregnancy (PK2, AP1, AP3)

247 GARCÍA, A.G. & SÁNCHEZ-GARCÍA, P. Influence of cocaine and sodium on bretylium uptake by reserpine-treated guinea-pig left atrium (PK3, PK2)

257 DA PRADA, M., OBRIST, R. & PLETSCHER, A. Discrimination of monoamine uptake by membranes of adrenal chromaffin granules (PK3, PK1)

Drug Mechanisms

267 KALSNER, S. Role of extraneuronal mechanisms in the termination of contractile responses to amines in vascular tissue (DM1, PK3, AP1)

279 CHIOU, C.Y. & MALAGODI, MARJORIE H. Studies on the mechanism of action of a new Ca^{2+} antagonist, 8-(*N*,*N*-diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride in smooth and skeletal muscles (DM1)

287 GREEN, A.R., SOURKES, T.L. & YOUNG, S.N. Liver and brain tryptophan metabolism following hydrocortisone administration to rats and gerbils (DM1, SP2)

293 HARRIS, D.W., SMY, J.R., REED, J.D. & VENABLES, C.W. The effects of burimamide and metiamide on basal gastric function in the cat (DM2, SP3, AP2)

Psychopharmacology

299 HOLMAN, R.B., HOYLAND, VALERIE & SHILLITO, ELIZABETH E. The failure of *p*-chlorophenylalanine to affect voluntary alcohol consumption in rats (PP, SP2)

SHORT COMMUNICATIONS

305 GREENWOOD, B. & KERRY, P.J. Prostaglandin production by a mild inflammatory lesion in sheep (AP2)

308 ADAMS, P.R. Kinetics of agonist conductance changes during hyperpolarization at frog endplates (DM1, DM2)

311 SCHILD, H.O. An ambiguity in receptor theory (DM2)

VOLUME 53 NUMBER 3 MARCH 1975

Systematic Pharmacology

317 CHAWLA, NARESH, JOHRI, M.B.L., SAXENA, P.N. & SINGHAL, K.C. Cholinergic mechanism in central thermoregulation in pigeons (SP2, AP1)

Autopharmacology

323 JONES, MARGARET E.L. & SPRIGGS, T.L.B. Noradrenaline and motor transmission in the vas deferens of the mouse (AP1)

333 MINATOYA, H. & SPILKER, B.A. Lack of cardiac or bronchodilator tachyphylaxis to isoprenaline in the dog (AP1)

341 BURDEN, D.T. & PARKES, M.W. An investigation of the tachycardia produced by intracerebroventricular injections of isoprenaline in mice (AP1, SP1, DM2)

349 SLOTKIN, T.A. Effects of reserpine on the adrenal medulla of the spontaneously hypertensive rat (AP1)

357 RUBIN, R.P. & WARNER, W. Nicotine-induced stimulation of steroidogenesis in adrenocortical cells of the cat (AP3, DM1)

page

Drug Mechanisms

363 ROBINSON, D.A., TAYLOR, J.G. & YOUNG, J.M. The irreversible binding of acetylcholine mustard to muscarinic receptors in intestinal smooth muscle of the guinea-pig (DM2, DM1)
371 HUGHES, J., KOSTERLITZ, H.W. & LESLIE, FRANCES M. Effect of morphine on adrenergic transmission in the mouse vas deferens. Assessment of agonist and antagonist potencies of narcotic analgesics (DM2)
383 COX, B.M., GINSBURG, M., WILLIS, JULIA & DAVIES, J.K. The offset of morphine tolerance in rats and mice (DM2, SP2)
393 NESHEIM, BRITT-INGJERD. Action of β-adrenoceptor antagonists on the response to isoprenaline in the oestrogen dominated rabbit uterus (DM2, AP1)
403 NESHEIM, BRITT-INGJERD, OSNES, J.-B. & ØYE, I. Role of cyclic adenosine 3′,5′-monophosphate in the isoprenaline-induced relaxation of the oestrogen dominated rabbit uterus (DM2, DM1, AP1)

Miscellaneous

409 EVANS, D.P. & THOMSON, D.S. Inhibition of immediate hypersensitivity reactions in laboratory animals by a phenanthroline salt (ICI 74,917) (M, AP2)

SHORT COMMUNICATIONS

419 ANLEZARK, G.M. & MELDRUM, B.S. Effects of apomorphine, ergocornine and piribedil on audiogenic seizures in DBA/2 mice (SP2, DM2, AP1)
422 BOWERY, N.G., BROWN, D.A. & COLLINS, J.F. Tetramethylenedisulphotetramine: an inhibitor of γ-aminobutyric acid induced depolarization of the isolated superior cervical ganglion of the rat (DM1, AP1)
425 AUNIS, D., MANDEL, P., MIRAS-PORTUGAL, M.T., COQUILLAT, G., ROHMER, F. & WARTER, J.M. Changes of human plasma dopamine-β-hydroxylase activity after intravenous administration of theophylline (DM1)
428 BOTTING, J.H. Sensitivity of neonatal rabbit ileum to histamine (AP2)

PROCEEDINGS OF THE BRITISH PHARMACOLOGICAL SOCIETY

London, 8-10 January, 1975

431P ENERO, MARIA, A. & LANGER, S.Z. Pharmacological effects of histamine on the isolated cat nictitating membrane.
432P HOLTON, PAMELA, IMPICCIATORE, MARIANINA & SPENCER, JAN. Dose response relationship and comparison of the secretory potency of methyl histamine and histamine on the isolated guinea pig stomach.
433P CURWAIN, B.P. & MILLS, G.A. Effects of atropine on gastric secretion and mucosal blood flow in conscious dogs.
434P McISAAC, R.L. & WILSON, G.M. (introduced by Pamela Holton). The effect of atropine on pentagastrin-induced gastric acid secretion and mucosal blood flow in the conscious rat.
435P BRIMBLECOMBE, R.W., DUNCAN, W.A.M., DURANT, G.J., GANELLIN, C.R., PARSONS, M.E. & BLACK, J.W. The pharmacology of cimetidine, a new histamine H_2-receptor antagonist.
436P FOSTER, R.W. & RAMAGE, A.G. Observations on the effect of dibenzoxazepine (CR) and N-nonoyl-vanillylamide (VAN) on sensory nerves.
437P CONSTANTI, A. & NISTRI, A. (introduced by Quilliam, J.P.). Actions of glutamate and kainic acid on the lobster muscle fibre and the frog spinal cord.
438P ADAMS, P.R. & BROWN, D.A. Actions of γ-aminobutyric acid (GABA) on ganglionic transmission and ganglion cell excitability.
439P DICK, FABIENNE & KELLY, J.S. L-2,4-diaminobutyric acid (L-DABA) as a selective marker for inhibitory nerve terminals in rat brain.

page

440P MEIER, J. (introduced by Berde, B.). Bioanalytical assay of clozapine and its N-oxide metabolite and the determination of their blood levels in the dog.
441P HEWICK, D.S. & MORELAND, T.A. (introduced by Marshall, P.BA). An NADPH dependent warfarin reductase in human and rat liver and kidney soluble fraction.
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443P AUTY, R.M. & BRANCH, R.A. (introduced by Bhoola, K.D.). The metabolism of ethyl, n-propyl, n-butyl and iso-amyl alcohols by the isolated perfused rat liver.
444P BENNETT, T. & GARDINER, SHEILA M. (introduced by Birmingham, A.T.). The effects of intravenous injections of vinblastine or vincristine on the responses of the rat heart to nerve stimulation and to drugs.
445P COTTNEY, J. & LEWIS, A.J. Mechanisms of concanavalin A-induced inflammation in the rat.
446P BLOGG, C.E., BRITTAIN, R.T., SIMPSON, B.R. & TYERS, M.B. AH 10407: A novel, short-acting, competitive neuromuscular blocking drug in animals and man.
447P ATTERWILL, C.K., MAHONEY, ANNE & NEAL, M.J. The uptake and subcellular distribution of ^{3}H-choline by the retina.
448P SCHILD, H.O. Drug desensitization and cross-desensitization in guinea-pig ileum.
448P TRIST, D.G. & WEATHERALL, M. The effect of isoprenaline on ^{86}Rb uptake by horse lymphocytes *in vitro.*
449P FOZARD, J.R. & MWALUKO, G.M.P. Stimulant effects of 5-hydroxytryptamine on cardiac sympathetic nerves.
450P GRAHAM, C.W., GREEN, A.R., WOODS, H.F. & YOUDIM, M.B.H. 5-Hydroxytryptamine synthesis in the isolated perfused rat brain.
451P LIVINGSTON, A. & PHILLIPS, ELIZABETH. Distribution of chlorpromazine and its metabolites in subfractions of rat brain.
452P TURNBULL, M.J. & WATKINS, J.W. Change in sensitivity to pentobarbitone and halothane induced by acute administration of central nervous system depressant drugs.
453P MOORE, K.E. & PHILLIPSON, O.T. (introduced by Beart, P.M.). Effects of dexamethasone on phenylethanolamine N-methyl-transferase (PNMT) and adrenaline (A) in the brains of adult and neonatal rats.
454P BRYANT, B.J., McCULLOCH, M.W., RAND, M.J. & STORY, D.F. Release of ^{3}H-(−)-noradrenaline from guinea-pig hypothalamic slices: effects of adrenoceptor agonists and antagonists.
455P DARGIE, H.J., DOLLERY, C.T. & LEWIS, P.J. Prevention of DOCA saline hypertension by central 6-hydroxydopamine: role of saline intake.
455P HIRSCH, J.C., TASSIN, J.P. & THIERRY, A.M. Loss of noradrenergic and dopaminergic terminals in the chronically isolated cerebral cortex of the cat.
456P ELKHAWAD, A.O., MUNDAY, K.A., POAT, JUDITH A. & WOODRUFF, G.N. The effect of dopamine receptor stimulants on locomotor activity and cyclic AMP levels in the rat striatum.
457P MILLER, R. (introduced by Iversen, L.L.). Inhibition of dopamine-sensitive adenylate cyclase in rat basal ganglia and other hormone sensitive adenylate cyclase systems by neuroleptic drugs.
458P CALLINGHAM, B.A. & LYLES, G.A. Some effects of age upon irreversible inhibition of cardiac MAO.
459P BEVAN, P., BRADSHAW, C.M. & SZABADI, E. Tricyclic antidepressants and monoamines: the relationship between uptake blockade and potentiation of neuronal responses.
460P AHTEE, LIISA. Effect of drugs on metoclopramide-induced catalepsy and increase in striatal homovanillic acid content.
461P BRIMBLECOMBE, R.W., BUXTON, D.A. & REDFERN, P.H. Drug effects on avoidance behaviour in selected strains of rats.
462P BRADLEY, P.B. & BRAMWELL, G.J. A stereospecific action of morphine on brain stem neuronal activity: a microiontophoretic study.
463P BENOY, CHRISTINE J., EL FELLAH, M.S., SCHNEIDER, R. & WADE, O.L. Tolerance to sympathomimetic bronchodilators in the guinea pig lungs.

F-98

464P JONES, R.L. Actions of prostaglandins on the arterial system of the sheep: some structure-activity relationships.
465P HERMAN, A.G. & MONCADA, S. Release of prostaglandins and incapacitation after injection of endotoxin in the knee joint of the dog.
466P PARRATT, J.R. & STURGESS, R.M. The protective effect of sodium meclofenamate in experimental endotoxin shock.
467P CRUTCHLEY, D.J. & PIPER, PRISCILLA J. Inhibition of the pulmonary inactivation of prostaglandins in rabbit *in vivo.*
467P BLACKWELL, G.J., FLOWER, R.J., PARSONS, M.F. & VANE, J.R. Factors influencing the turnover of prostaglandin synthetase.
469P PUGH, D.M., SHARMA, S.C. & WILSON, C.W.M. Inhibitory effect of L-ascorbic acid on the yield of prostaglandin F from the guinea-pig uterine homogenates.
470P GREAVES, M.W., KINGSTON, W.P. & PRETTY, KATHLEEN. Action of a series of non-steroid and steroid anti-inflammatory drugs on prostaglandin synthesis by the microsomal fraction of rat skin.

VOLUME 53 NUMBER 4 APRIL 1975

473 SNYDER, S.H. (work done in collaboration with Anne B. Young). Fifth Gaddum Memorial Lecture, University of Bristol, September 1974. The glycine synaptic receptor in the mammalian central nervous system (AP1, DM2, SP2)

Systematic Pharmacology

485 PARRATT, J.R. & STURGESS, R.M. *E. coli* endotoxin shock in the cat; treatment with indomethacin (SP1)
489 JOHNSON, K.G. Thermoregulatory changes induced by cholinomimetic substances introduced into the cerebral ventricles of sheep (SP2, AP1)
499 DE MICHELI, P. & GLÄSSER, A.H. The effects of catecholamines and adrenoceptor blocking drugs on the canine peripheral lymph flow (SP3, AP1, DM2)

Autopharmacology

505 HENDERSON, G., HUGHES, J. & KOSTERLITZ, H.W. The effects of morphine on the release of noradrenaline from the cat isolated nictitating membrane and the guinea-pig ileum myenteric plexus-longitudinal muscle preparation (AP1, DM2, PK3)
513 PACHA, W., SALZMANN, R. & SCHOLTYSIK, G. Inhibitory effects of clonidine and BS 100-141 on responses to sympathetic nerve stimulation in cats and rabbits (AP1)
517 DAWES, P.M. & FAULKNER, D.C. The effect of propranolol on vascular responses to sympathetic nerve stimulation (AP1, DM2, SP1)
525 HOOD, A.J.C., SMY, J.R. & WEETMAN, D.F. An indirect sympathomimetic effect of burimamide on kitten isolated atria (AP1, AP2, SP1)
531 CHAMALES, M.H., GOURLEY, R.D. & WILLIAMS, BETTY J. Effect of acetylcholine on changes in contractility, heart rate and phosphorylase activity produced by isoprenaline, salbutamol and aminophylline in the perfused guinea-pig heart (AP1, DM2, SP1)
539 HIGGS, G.A., McCALL, ELAINE & YOULTEN, L.J.F. A chemotactic role for prostaglandins released from polymorphonuclear leucocytes during phagocytosis (AP2)
547 BEDWANI, J.R. & MARLEY, P.B. Enhanced inactivation of prostaglandin E_2 by the rabbit lung during pregnancy or progesterone treatment (AP2, AP3)
555 HODGES, J.R. & VELLUCCI, SANDRA V. The effect of reserpine on hypothalamo-pituitary-adrenocortical function in the rat (AP3)

page

Pharmacokinetics

563 BUCZKO, W., DE GAETANO, G. & GARATTINI, S. Effect of fenfluramine on 5-hydroxytryptamine uptake and release by rat blood platelets (PK3, DM1)
569 FANTOZZI, R., FRANCONI, FLAVIA, MANNAIONI, P.F., MASINI, EMANUELA & MORONI, F. Interaction of histamine H_1- and H_2-receptor antagonists with histamine uptake and metabolism by guinea-pig isolated atrium and mouse neoplastic mast cells *in vitro* (PK2, PK3, DM2)

Drug Mechanisms

575 SPEDDING, M., SWEETMAN, A.J. & WEETMAN, D.F. Antagonism of adenosine 5′-triphosphate-induced relaxation by 2-2′-pyridylisatogen in the taenia of guinea-pig caecum (DM1, DM2)
585 KENNEDY, I. & LEVY, G.P. Combined α- and β-adrenoceptor blocking drug AH 5158: further studies on α-adrenoceptor blockade in anaesthetized animals (DM2)

SHORT COMMUNICATIONS

593 el-AZHARY, ROKEA A., KHAYYAL, M.T. & SALEH, SAMIRA. Effect of fluphenazine on tissue noradrenaline concentrations and its interaction with pargyline (SP2, PP, PK2)
596 GREENBERG, R. Indomethacin potentiation of the response of the rabbit portal vein to electrical stimulation: effect of stimulus frequency and train length (AP1, AP2)
598 LARGE, W.A. Effect of tetramethylenedisulphotetramine on the membrane conductance increase produced by γ-amino-butyric acid at the crab neuromuscular junction (DM1)

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British Journal of Pharmacology

VOLUME 53 NUMBER 4 APRIL 1975

page

473 SNYDER, S.H. (work done in collaboration with Anne B. Young). Fifth Gaddum Memorial Lecture, University of Bristol, September 1974. The glycine synaptic receptor in the mammalian central nervous system

Systematic Pharmacology

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